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(54) **Title**: ANTICD20-CPG CONJUGATES AND METHODS OF TREATING B CELL MALIGNANCIES

(57) **Abstract**: Disclosed herein are antiCD20-CpG conjugates and methods of using thereof to treat or inhibit B cell malignancies.

ANTICD20-CPG CONJUGATES AND METHODS OF TREATING B CELL MALIGNANCIES

CROSS REFERENCE TO RELATED APPLICATIONS

[01] This application claims the benefit of U.S. Provisional Patent Application Serial No. 61/178,366, filed 14 May 2009, which is herein incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

1. FIELD OF THE INVENTION.

[02] The present invention generally relates to anti-CD20 monoclonal antibody therapies.

2. DESCRIPTION OF THE RELATED ART.

[03] Anti-CD20 monoclonal antibody therapy (e.g. rituximab) is a cornerstone in the treatment of B cell non-Hodgkin's lymphomas. Rituximab therapy, however, is only partially effective, as most patients eventually relapse. As the primary mechanism of rituximab's action is antibody-dependent cellular cytotoxicity (ADCC) via natural killer (NK) cells and macrophages, combination therapies with various immunostimulants capable of activating these effectors have been attempted in both animal and human studies.

[04] Rituximab binds to the CD20 antigen, a 33-35 kDa transmembrane protein expressed on the surface of normal B cells and over 90% of B cell non-Hodgkin's lymphomas (NHL). Rituximab is often added to conventional chemotherapy regimens to treat NHL in subjects. Unfortunately, resistance to rituximab eventually occurs in most subjects, usually despite the continued expression of CD20 by tumor cells, and leads to a large incidence of death.

[05] The *in vivo* anti-lymphoma effects of rituximab may result from several possible mechanisms, including direct anti-proliferative and/or pro-apoptotic signaling, complement-dependent cytotoxicity (CDC), and antibody-dependent cellular cytotoxicity (ADCC) by Fc receptor (FcR)-bearing effector cells. ADCC appears to play the dominant role *in vivo*, based on studies in FcyR-deficient mice and clinical studies in humans. See Clynes et al. (2000) Nat Med.6:443-446; Cartron et al. (2002) Blood 99:754-758; and Weng et al. (2003) J Clin Oncol. 21:3940-3947. A fourth possible mechanism for rituximab action involves the secondary recruitment of

anti-tumor T cell effectors responding to tumor antigens released upon antibody-mediated tumor lysis via the process of "cross-priming". See Selenko et al. (2001) Leukemia 15:1619-1626; Dhodapkar et al. (2002) J Exp Med. 195:125-133; Groh et al. (2005) PNAS USA 102:6461-6466; Dhodapkar (2005) PNAS USA 102:6243-6244; and Franki et al. (2008) Blood 111:1504-1511.

[06] Oligonucleotides containing unmethylated cytosine-guanine (CpG) dinucleotides capable of activating leukocytes as evidenced *in vitro* and *in vivo* are referred to as immunostimulatory "CpG" oligodeoxynucleotides (ODN). These immunostimulatory ODN are synthetic, are about 2 to 100 base pairs in length, and contain a consensus unmethylated CpG motif. The immune enhancing properties of CpGs are under study for their ability to stimulate anti-tumor immunity. See Kanzler et al. (2007) Nat Med. 13:552-559; and Krieg et al. (2007) 117:1184-1194.

[07] Systemic administration of CpG (subcutaneous; s.c., or intravenous; i.v.) has been studied in clinical trials for B cell lymphoma either alone, or in combination with rituximab. See Link et al. (2006) J Immunother. 29:558-568; Friedberg et al. (2005) Blood 105:489-495; and Leonard et al. (2007) Clin Cancer Res. 13:6168-6174. Unfortunately, systemically-administered CpG does not appear to have significant single-agent activity against B cell NHL, or the capacity to substantially enhance the efficacy of rituximab. See Link et al. (2006) J Immunother. 29:558-568; Friedberg et al. (2005) Blood 105:489-495; and Leonard et al. (2007) Clin Cancer Res. 13:6168-6174.

[08] Recent studies suggest that local, intratumoral injection of CpG is promotes anti-tumor immunity. See Heckelsmiller et al. (2002) J Immunol. 169:3892-3899; Sharma et al. (2003) Biotechnol Lett. 25:149-153; Lonsdorf et al. (2003) J Immunol. 171:3941-3946; Furumoto et al. (2004) J Clin Invest. 113:774-783; and Sharma et al. (2008) Cancer Immunol Immunother. 57:549-561. In mice, injection of CpG directly into tumor sites after chemotherapy-mediated killing leads to cure of bulky, established lymphoma, and subsequent systemic anti-lymphoma immunity. See Li et al. (2007) J Immunol. 179:2493-2500. A recent clinical trial showed irradiation of a single peripheral lymphoma site followed by intratumoral injection with human CpG 7909 can lead to regression of distant tumor metastases. See Brody et al. (2008) J Clin Oncol 26: 2008 (May 20 suppl; abstr 3003).

[09] Unfortunately, intratumoral injection is not feasible for most patients with B cell malignancies, as tumors either reside deep within the body, or as multiple small deposits within tissues.

SUMMARY OF THE INVENTION

[10] The present invention provides an antiCD20-CpG conjugate which comprises an antiCD20 portion linked with a linker to a CpG molecule. Suitable CpGs according to the present invention are preferably stabilized oligonucleotides, e.g. phosphorothioate stabilized oligonucleotides, which are relatively resistant to *in vivo* degradation, using methods known in the art. In some embodiments, the linker is a cleavable linker. In some embodiments, the antiCD20 portion is an anti-CD20 antibody or a molecule which specifically binds CD20. In some embodiments, the CpG molecule is a class B CpG. In some embodiments, the CpG molecule is CpG 1826, CpG 10103, or CpG 7909. In some embodiments, the CpG molecule comprises one or more of the following sequences $[CGN_a]_x$, $[N_aCG]_x$, $[N_aCGN_b]_x$, $[N_aCGTTN_b]_x$, and $[N_aCGN_bCGN_c]_x$, where N is any nucleotide base, x is 0-25 and a, b and c are independently 1-15. In some embodiments, the antibody is rituximab, ocrelizumab, PRO131921, GA101, tositumomab, ofatumumab, veltuzumab, AME-133, TRU-015, or 1F5. In some embodiments, the antiCD20-CpG conjugate is rituximab-CpG 1826, rituximab-CpG 10103, or rituximab-CpG 7909.

[11] In some embodiments, an antiCD20-CpG conjugate of the present invention is provided in a pharmaceutical composition comprising a pharmaceutically acceptable carrier or excipient. In some embodiments, the antiCD20-CpG conjugate is packaged in a kit as a single dose or in multiple doses. The single dose may be a therapeutically effective amount or the multiple dose may together equal a therapeutically effective amount. Similarly, the single dose may be a maintenance effective amount or the multiple dose may together equal a maintenance effective amount.

[12] In some embodiments, the present invention provides a kit which comprises an antiCD20-CpG conjugate as described herein packaged together with one or more of the following: instructional material, a device to administer the antiCD20-CpG conjugate, and the like.

[13] In some embodiments, the present invention provides a method for treating or inhibiting a B cell malignant malignancy in a subject which comprises administering

to the subject an amount of an antiCD20-CpG conjugate according to the present invention. In some embodiments, the amount administered is a therapeutically effective amount. In some embodiments, the B cell malignancy is a CD20-positive B cell malignancy. In some embodiments, the antiCD20-CpG conjugate is systemically administered to the subject. In some embodiments, the antiCD20-CpG conjugates are administered as a pharmaceutical composition.

[14] Both the foregoing general description and the following detailed description are exemplary and explanatory only and are intended to provide further explanation of the invention as claimed. The accompanying drawings are included to provide a further understanding of the invention and are incorporated in and constitute part of this specification, illustrate several embodiments of the invention, and together with the description serve to explain the principles of the invention.

DESCRIPTION OF THE DRAWINGS

[15] This invention is further understood by reference to the drawings wherein:

[16] Figure 1 schematically shows the anti-tumor mechanisms of anti-CD20 monoclonal antibodies and CpGs. Anti-CD20 antibodies such as rituximab bind to the CD20 antigen on the surface of malignant B cells and have 4 demonstrated mechanisms of cell killing: 1) inhibition of cellular proliferation and/or induction of apoptosis via intracellular signaling events, 2) activation of the complement cascade to mediate complement-dependent cytotoxicity, 3) antibody-dependent cellular cytotoxicity via Fc receptors (FcR) on natural killer cells, macrophages, and neutrophils, and 4) induction of T cell anti-tumor immunity to tumor antigens uptaken and presented by dendritic cells (DC) after antibody-mediated killing. CpGs can enhance the killing of malignant B cells via amplification of each antibody killing mechanisms.

[17] Figure 2 is a graph showing that established s.c. 38C13-huCD20 tumors are resistant to single agent rituximab. Mice were injected s.c. with 5×10^3 tumor cells on day 0. A single 500 mg i.v. injection of rituximab was administered either on day 0, 1, 3, 5, 7, or 10, and mice followed for survival. Data are representative of 2 independent experiments.

[18] Figure 3A is a graph showing intratumoral injection of CpG is required for rituximab-mediated eradication of established 38C13-huCD20 lymphoma. Groups of 12 mice were injected s.c. with 5×10^3 38C13-huCD20 tumor cells on day 0. On days

7 and 9 mice received 500 mg of rituximab i.v. CpG was injected i.t. or i.p. with 50 mg given on days 7, 8 and 9, and 25 mg given on days 11, 13, and 15. B) Control antibody and ODN have no effects on tumor growth *in vivo*. Groups of 12 mice were injected s.c. with 5 x 10^3 38C13-huCD20 tumor cells on day 0. On days 7 and 9 mice received 500 mg of either rituximab or the irrelevant isotype-matched control antibody trastuzumab i.v. The ODNs were injected i.t. with 50 mg given on days 7, 8 and 9, and 25 mg given on days 11, 13, and 15. Data are representative of 3 independent experiments.

[19] Figure 3B is a graph showing intratumoral injection of CpG is required for rituximab-mediated eradication of established 38C13-huCD20 lymphoma. Control antibody and ODN have no effects on tumor growth *in vivo*. Groups of 12 mice were injected s.c. with 5 x 10^3 38C13-huCD20 tumor cells on day 0. On days 7 and 9 mice received 500 mg of either rituximab or the irrelevant isotype-matched control antibody trastuzumab i.v. The ODNs were injected i.t. with 50 mg given on days 7, 8 and 9, and 25 mg given on days 11, 13, and 15. Data are representative of 3 independent experiments.

[20] Figure 4 is a graph showing a single injection of rituximab-CpG 1826 conjugate eradicates 5-day established human CD20+ lymphoma. Groups of 12 mice were inoculated subcutaneously with 5 x 10^3 38C13-huCD20 tumor cells on day 0. After 5 days, mice were then treated (Rx) with a single intravenous injection of SMCC rituximab-CpG conjugate (211 μg Rituximab:50 μg CpG), rituximab alone (211 μg), CpG alone (50 μg), or a mixture of identical doses of rituximab and CpG. Mice were followed for survival and sacrificed when tumors reached 1.4 cm. *Indicates one mouse died with no palpable tumor.

[21] Figure 5 is a graph showing that rituximab-CpG 1826 conjugate eradicates 7-day established human CD20+ lymphoma with antigen and CpG specificity. Groups of 12 mice were inoculated subcutaneously with 5 x 10^3 38C13-huCD20 tumor cells on day 0. After 7 days, mice were then treated with 2 i.v. injections (days 7 & 10; Rx) of rituximab-CpG conjugate, rituximab mixed with CpG, rituximab and intratumoral (i.t.) CpG, rituximab conjugated to control ODN, control antibody (trastuzumab) conjugated to CpG or control ODN, or control HBSS (saline). CpG dose was 50 μg, with conjugate doses normalized to contain 50 μg of CpG (about 200 μg antibody). Mice were followed for survival and sacrificed when tumors reached 1.4 cm.

[22] Figure 6 is a graph showing tumor eradication is dependent upon natural killer cells and complement. Groups of 8 mice were injected s.c. with 5 x 10^3 38C13-huCD20 tumor cells on day 0. To deplete natural killer cells, mice were injected i.p. with 50 ml of asialo-GM1 on days 1, 6, and 11. To deplete complement, mice were injected i.p. with 25 U of cobra venom factor on days 1, 4, 7, 10 and 13. Mice were treated with rituximab-CpG 1826 conjugate i.v. (normalized to 50 mg of CpG) on days 7 and 10. Mice were followed for survival and sacrificed when tumors reached 1.4 cm in diameter.

[23] Figure 7 is a graph showing that the role for macrophages in lymphoma eradication by antiCD20-CpG is little. Groups of 8 mice were injected s.c. with 5 x 10^3 38C13-huCD20 tumor cells on day 0. To deplete macrophages, mice were injected i.p. with 150 ml of clodronate or saline encapsulated in liposomes on days 1, 4, 7, 10, and 13. Mice were treated with rituximab-CpG 1826 conjugate i.v. (normalized to 50 mg of CpG) on days 7 and 10. Mice were followed for survival and sacrificed when tumors reached 1.4 cm in diameter.

[24] Figure 8 is a graph showing that T cells have no role in initial tumor eradication by antiCD20-CpG. Groups of 8 mice were injected s.c. with 5 x 10^3 38C13-huCD20 tumor cells on day 0. To deplete T cell subsets, mice were injected i.p. with 200 mg of monoclonal antibodies HB129 (anti-CD8), or GK1.5 (anti-CD4), or control antibodies on days 1, 2, 3, 6, and weekly thereafter for the duration of the experiment. Depletion of CD4+ and CD8+ T cells was verified by flow cytometric analysis of peripheral blood on day 6. Mice were treated with with rituximab-CpG 1826 conjugate i.v. (normalized to 50 mg of CpG) on days 7 and 10. Mice were followed for survival and sacrificed when tumors reached 1.4 cm in diameter.

[25] Figure 9 is a graph showing that type 1 interferon plays a little role in tumor eradication by antiCD20-CpG. Groups of 8 mice were injected s.c. on day 0 with 5 x 10^3 38C13-huCD20 cells or 38C13-huCD20 cells rendered deficient in interferon alpha receptor (IFNAR-/-) by lentiviral transduction with shRNA against IFNAR. These latter cells are insensitive to the growth inhibitory effects of type I (alpha and beta) IFNs. Mice were treated with rituximab-CpG 1826 conjugate i.v. (normalized to 50 mg of CpG) on days 7 and 10. Mice were followed for survival and sacrificed when tumors reached 1.4 cm in diameter.

[26] Figure 10A is a graph showing oligodeoxynucleotides (ODN) inhibit the proliferation of human B cell lymphoma cells. Human Daudi B cell lymphoma cells

were cultured in the presence of media alone, or with human class B CpG 10103, human control ODN 2137, mouse class B CpG 1826, or its control ODN 2138. After overnight culture, proliferation was measured by 8 hour pulse with 3H-thymidine. Concentration-dependent growth inhibitory effects are seen with both human and murine CpGs. Data are represented as mean +/- standard deviation of quadruplicate values.

[27] Figure 10B provides graphs demonstrating that CpGs of different classes directly inhibit the proliferation of various aggressive human B cell lymphomas. These include Raji (Burkitt's lymphoma), OCI-Ly19 (germinal center type diffuse large B cell lymphoma (GCB-DLBCL)), SU-DHL-2 (activated B cell type diffuse large B cell lymphoma (ABC-DLBCL)), and Granta 519 (mantle cell lymphoma). Cells were plated 10,000 cells/well in 5 replicates in 96-well U-bottom plates with media alone, or with class A (2336), B (7909), or C (2395) CpGs or control ODNs (2243, 2137) at (2, 10, or 50 μg/ml). Plates were incubated for 72 hours, then ^{3}[H]-thymidine pulsed for 8 hours. Data are represented as mean +/- standard deviation of replicate values. Class B CpG and ODN have the most potent anti-proliferative effects.

[28] Figure 11 schematically shows SMCC antibody-CpG conjugation chemistry. Sulfo-SMCC forms an amide bond after reacting with the 5'-amino-modified CpG, while the maleimide group forms a cleavable bond with the free sulfhydryl group on the hinge region of the antibody. The sequence shown is SEQ ID NO:3.

[29] Figure 12 is a graph showing intact biologic activity of CpG coupled to rituximab. Sulfo-SMCC-activated CpG 1826 was linked to partially-reduced rituximab, and unbound CpG removed by ultrafiltration. A20-huCD20 lymphoma cells were cultured for 48 hours with media alone, 10 μg/ml CpG, or rituximab-CpG conjugate (with 10 μg/ml CpG content). CpG bioactivity was measured by the ability to upregulate expression of the costimulatory molecule ICAM-1/CD54. Flow cytometric analysis demonstrates equivalent levels of ICAM-1 upregulation by free CpG and the antibody conjugate, indicating full bioactivity.

[30] Figure 13 schematically shows MHPH/4FB antibody-CpG conjugation chemistry. The sequence shown is SEQ ID NO:3.

[31] Figure 14 are graphs showing the activation of dendritic cells by rituximab-CpG conjugates. Bone marrow-derived dendritic cells were cultured overnight with media alone, 10 μg/ml CpG 1826, rituximab, or MHPH/4FB rituximab-CpG

conjugate (with 10 μg/ml CpG content). Expression of CD80/B7.1, CD86/B7.2, and MHC class II was measured by flow cytometry. Mean fluorescence intensity for each marker is indicated in the right upper corner of each panel.

DETAILED DESCRIPTION OF THE INVENTION

[32] The present invention provides antiCD20-CpG conjugates. According to the present invention, an antiCD20-CpG conjugate is an anti-CD20 antibody or a functionally equivalent molecule linked to a CpG. As used herein, a "CpG" is a nucleic acid molecule containing one or more cytosines followed by a guanosine and linked by a phosphate bond. In preferred embodiments, the CpGs exhibit Toll-Like Receptor 9 (TLR9) agonist activity. See Kanzler et al. (2007) Nat Med. 13:552-559; and Krieg et al. (2007) J Clin Invest. 117:1184-1194, which are herein incorporated by reference. TLR9 agonist activity can be detected and measured using methods known in the art, e.g. by incubation with HEK-Blue-hTLR9 cells (InvivoGen, Inc., San Diego, CA). Delivery of CpG into a tumor bed has the potential to boost all 4 mechanisms of action, shown in Figure 1, of anti-CD20 antibodies against B cell malignancies.

[33] The present invention also provides methods of treating B cell malignancies, preferably CD20-positive B cell malignancies, in subjects by administering to the subject a therapeutically effective amount of antiCD20-CpG conjugate. CD20-positive B cell malignancies include Chronic lymphocytic leukemia/Small lymphocytic lymphoma, B-cell prolymphocytic leukemia, Lymphoplasmacytic lymphoma (Waldenström macroglobulinemia), Splenic marginal zone lymphoma, Plasma cell myeloma, plasma cell leukemia, Plasmacytoma, Monoclonal immunoglobulin deposition diseases, Extranodal marginal zone B cell lymphoma (MALT lymphoma, Nodal marginal zone B cell lymphoma (NMZL), Follicular lymphoma, Mantle cell lymphoma, Diffuse large B cell lymphoma, Mediastinal (thymic) large B cell lymphoma, Intravascular large B cell lymphoma, Primary effusion lymphoma, Burkitt lymphoma/leukemia, Classical Hodgkin lymphomas (Nodular sclerosis; Mixed cellularity; Lymphocyte-rich; Lymphocyte depleted or not depleted), Nodular lymphocyte-predominant Hodgkin lymphoma, Immunodeficiency-associated lymphoproliferative disorders (including those associated with a primary immune disorder, associated with the Human Immunodeficiency Virus (HIV), post-transplant, or associated with Methotrexate therapy).

[34] Prior art models using human lymphoma xenografts in SCID/nude mice have severe limitations, in that they do not allow for (1) physiologic tumor growth in a natural host, where tumor cell adhesion, homing/metastasis, and cytokine/chemokine networks are fully functional, or (2) secondary recruitment of adaptive T cell immune effector mechanisms. Unlike prior art models using human lymphoma xenografts in SCID/nude mice, the huCD20 models described herein allow tumor cell growth and trafficking in the tumor's natural host species and the opportunity to enhance host innate and secondary adaptive immune responses against tumor antigens released following antibody-mediated cytotoxicity.

[35] As described herein, murine lymphoma models expressing human CD20 (huCD20 models) were used to develop and study anti-CD20 monoclonal antibody combination therapies for treating B cell malignancies. In particular, 38C13 cells transduced with the gene encoding human CD20 (38C13-huCD20) were used. See Golay et al. (2006) Haematologica 91:176-183, which is herein incorporated by reference. The cell line 38C13-huCD20, a murine B cell lymphoma engineered to express the human CD20 antigen, was used as a model of human B cell lymphomas expressing CD20 antigen. 38C13-huCD20 cells express on their surface high levels of CD20 comparable to that expressed by human B cell lymphomas. The 38C13 lymphoma is a highly aggressive tumor, with which micrometastases can be detected in the spleen, lymph nodes, and bone marrow of mice bearing s.c. tumors within 6-9 days of inoculation. See Neeson et al. (2004) Cytometry A 60:8-20, which is herein incorporated by reference. Despite the high level of CD20 expression, these cells are relatively resistant to the anti-CD20 antibody rituximab after 3 or more days of *in vivo* growth in mice. Thus, 38C13-huCD20 cells are a suitable cell model of human B cell lymphomas.

[36] As shown in Figure 2, rituximab alone is capable of eradicating 38C13-huCD20 tumor cells early after their introduction into the tissues (0-1 days), but not after 3 or more days of establishment. Tumor clearance in this model was found to be mediated by a combination of enhanced ADCC, CDC, and direct anti-proliferative effects. Thus, intratumoral injection of free CpG directly into the tumor may inhibit tumor growth in four ways: (1) increased sensitivity to complement lysis, (2) increased sensitivity to ADCC lysis, (3) increased cytotoxicity of ADCC effectors, and (4) direct anti-proliferative effects.

[37] Despite the resistance of the human CD20+ 38C13-huCD20 tumor to rituximab, it was found that direct intratumoral (i.t.) injection of a CpG, but not systemic delivery, somewhat enhanced the ability of rituximab to eradicate 7-day established tumors (Figure 3) (42% vs. 0%, $p<0.0003$). Specifically, mice were injected s.c. on the hind flank with 5 x 10^3 38C13-huCD20 tumor cells in 1X Hank's buffered saline solution (HBSS) on day 0, and sacrificed when tumors reached 1.4 cm in diameter per institutional guidelines. Mice were treated i.v. with 500 μg of rituximab or trastuzumab on days 7 and 9 unless otherwise indicated. CpG 1826 (5'-TCCATGA**CG**TTCCTGACGTT; bold nucleotides represent the immunostimulatory CpG sequences) and control CpG 2138 (5'-TCCATGAGCTTCCTGAGCTT) were purchased from Coley Pharmaceuticals Group (Wellesley, MA). Free CpGs were injected i.t., intraperitoneally (i.p.), or s.c. on days 7, 8, and 9 at 50 μg per dose, then at 25 μg per dose on days 11, 13, and 15. The i.p. route was chosen for systemic delivery of CpG. Injections were administered using a 27G needle in a volume of 100 μl 1X phosphate buffered saline (PBS).

[38] Thus, administration of an anti-CD20 antibody plus intratumoral administration of a free CpG (anti-CD20 + i.t.CpG) is necessary for achieving tumor eradication in rituximab-resistant lymphoma.

[39] Unexpectedly, however, it was discovered that systemic administration of an anti-CD20 monoclonal antibody directly linked to a CpG (antiCD20-CpG conjugate) exhibits far superior efficacy against established aggressive B cell lymphomas *in vivo* as compared treatment with rituximab alone, free CpG alone, or a simple mixture of rituximab plus free CpG.

[40] Specifically, as shown in Figure 4, rituximab alone, free CpG alone, or a simple mixture of rituximab plus free CpG cured only 1 of 12 tumor-bearing mice of 5-day established tumors. In contrast, no tumors grew in mice treated with the antiCD20-CpG conjugate and the antiCD20-CpG conjugate was able to reproducibly eradicate established tumors from 100% of mice bearing a highly aggressive human CD20+ B cell lymphoma resistant to native rituximab. Therefore, the antiCD20-CpG conjugates according to the present invention exhibit potent *in vivo* activity against human CD20+ B cell malignancies and may be used to treat or inhibit CD20+ B cell malignancies.

[41] Similarly, as shown in Figure 5, treatment with rituximab-CpG conjugate was able to eradicate 100% of 7-day established 38C13-huCD20 tumors (*P*-value = 0.0001

vs. HBSS or rituximab-2137 ODN conjugate), while rituximab alone, control ODN, and control conjugates had no significant effect on tumor growth. While equivalent doses of CpG administered intravenously with rituximab, or injected directly into the tumor site did slow tumor growth somewhat, neither cured any animals of their tumors. Thus, the present invention provides antiCD20-CpG conjugates and methods of making and using to treat or inhibit CD20+ B cell malignancies.

[42] It was found that both natural killer cells and complement participated in the eradication of lymphomas by the antiCD20-CpG conjugates according to the present invention. However, the T cells, macrophages, and alpha-interferon did not play any significant role in mediating tumor eradication by the antiCD20-CpG conjugate.

[43] Specifically, i*n vivo* depletion studies were performed to identify the key immunologic effectors responsible for tumor eradication by rituximab-CpG 1826 conjugate. As shown in Figure 6, depletion of NK cells completely abrogated tumor eradication by the rituximab-CpG 1826 conjugate, thereby indicating a critical role for NK cell-mediated ADCC in tumor killing using this therapy. Also as shown in Figure 6, depletion of complement using cobra venom factor also nearly completely abolished the anti-tumor effects of the rituximab-CpG 1826 conjugate.

[44] Macrophages have been reported to be important effectors in the clearance of normal B cells from mice treated with anti-CD20 monoclonal antibodies. See Uchida et al. (2004) J. Exp. Med. 199:1659-1669, which are herein incorporated by reference. Nonetheless, as shown in Figure 7, depletion of macrophages from mice treated with rituximab-CpG did not result in significant loss of tumor protection compared to controls (100% vs. 63%, respectively, PBS liposomes vs. clodronate lipsomes $P = 0.063$). Moreover, as shown in Figure 8, depletion of either $CD4^+$ or $CD8^+$ T cells did not reduce tumor-free survival compared to controls, thereby indicating that adaptive T cell immunity did not contribute to elimination of tumor during rituximab-CpG conjugate therapy.

[45] As shown in Figure 9, tumor cells deficient in alpha-IFN receptor were nearly as sensitive to the rituximab-CpG 1826 conjugate as IFNAR+ 38C13-huCD20 cells (*P*-value 0.26), indicating no significant role in eradication of 38C13-huCD20 tumors. Overall, these results demonstrate roles for NK cells and complement in the eradication of established 38C13-huCD20 tumors by the rituximab-CpG 1826 conjugate.

[46] Also unexpectedly, it was found that CpGs can directly inhibit the proliferation of human B cell non-Hodgkin lymphoma cells. While normal B cells are stimulated by CpGs, the effects of CpGs on malignant B cells are variable. CpG 1826 has potent anti-proliferative effects against the A20 murine B cell line, although direct growth inhibitory effects against 38C13 lymphomas are very modest. To determine if CpGs have similar inhibitory effects against human lymphomas, the direct effects of free CpGs on the human B cell non-Hodgkin lymphoma lines Raji, OCI-Ly19, SU-DHL-2, and Granta 519 were examined. As shown in Figure 10B, class B CpGs inhibited tumor cell proliferation by 25-50% in all cases, with class A and C CpGs having lesser effects. Curiously, class B control ODN 2137 also had inhibitory effects as compared to media controls.

[47] As disclosed herein, SMCC antiCD20-CpG conjugates were formed by coupling a modified 5'-amino-CpG to reduced hinge region sulfhydryl groups of rituximab using sulfhydryl-maleimide chemistry to generate a cleavable linker. See Betting et al. (2008) 181:4131-4140; and WO 2007106435, which are herein incorporated by reference. The SMCC antiCD20-CpG conjugates retained the CD20 binding properties of the antibody and the biologic activity of the CpG. Therefore, in some embodiments, any cleavable linker which allows retention of the CD20 binding properties of the antibody and the biologic activity of the CpG and methods for conjugation known in the art may be used in accordance with the present invention.

[48] In some embodiments, the cleavable linker reacts with reduced sulfhydryl groups in the hinge region or elsewhere within the anti-CD20 antibody via a maleimide group. The linker system is heterobifunctional in that the opposite end reacts not with sulfhydryl groups, but with amines or other reactive groups with the CpG. Some embodiments of the invention are based upon the discovery that one or more free sulhydryl groups on an antibody can be used to form a cleavable linkage, e.g., a thioether linkage, that covalently links the antibody to another biomolecule such as CpG. The compounds made according to this protocol have surprisingly enhanced efficacy when used to treat CD20+ B cell lymphoma as compared to antibodies not bearing CpG. An example of such a linker system is sulfosuccinimidyl-4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (sulfo-SMCC, Pierce, Rockford, IL). Other linkers that can be used in practicing the invention readily can be found in the scientific literature as well as in supply catalogs from Pierce Biotechnology, Rockford, IL (www.piercenet.com). These include: Sulfo-

GMBS (N-[g-Maleimidobutyryloxy]sulfosuccinimide ester), Sulfo-MBS (m-Maleimidobenzoyl-N-hydroxysulfosuccinimide ester), Sulfo-LC-SMPT (4-Sulfosuccinimidyl-6-methyl-a-(2-pyridyldithio)toluamido]hexanoate)), Sulfo-EMCS ([N-e-Maleimidocaproyloxy]sulfosuccinimide ester), EMCA (N-e-Maleimidocaproic acid), BMPA (N-ß-Maleimidopropionic acid), Sulfo-SIAB (N-Sulfosuccinimidyl[4-iodoacetyl]aminobenzoate), Sulfo-SMPB (Sulfosuccinimidyl 4-[p-maleimidophenyl]butyrate), Sulfo-LC-SPDP (Sulfosuccinimidyl 6-(3'-[2-pyridyldithio]-propionamido)hexanoate), Sulfo-KMUS (N-[k-Maleimidoundecanoyloxy]sulfosuccinimide ester), and the like.

[49] In some embodiments of the invention, bis-arylhydrazone chemistry is used to form the cleavable linker with cross-linking agents known in the art and include those available from SoluLink, San Diego, CA. Some cross-linking systems include a pair of reagents that yields a cleavable linkage between protein and oligonucleiotide such as S-SS-4FB (C6-Succinimidyl 4-formylbenzoate) and MHPH (3-N-Maleimido-6-hydraziniumpyridine hydrochloride).

[50] In some embodiments the linker is cleavable under *in vivo* conditions, thereby permitting release of CpG within or near tumor cells. The released CpG then inhibits growth by direct action of malignant B cells, by amplifying antibody cytotoxicity mechanisms, or by stimulating a host anti-tumor immune response.

[51] In some embodiments, the linkers of antiCD20-CpG conjugates of the present invention are not be considered to be cleavable based on current scientific knowledge. Such linkers include EDC (1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide Hydrochloride), from Pierce, Inc. (Rockford, IL). Such non-cleavable antiCD20-CpG conjugates are still advantageous as they may provide targeted delivery of biologically active CpGs to tumor sites by linkage to the anti-CD20 portion.

[52] In some embodiments, the antiCD20-CpG conjugates of the present invention may be linked by using strepavidin-biotin binding. In these embodiments, a CpG of interest is chemically biotinylated, then bound to streptavidin which is either fused or chemically linked to the anti-CD20 portion.

[53] The anti-CD20 portion of the conjugate is an anti-CD20 antibody that specifically binds the CD20 differentiation antigen found on B cell malignancies. In some embodiments, the anti-CD20 antibody is a monoclonal antibody. In some embodiments, the anti-CD20 portion of the conjugate is a fragment of an anti-CD20 antibody or a recombinant derivative thereof, or a molecule that is functionally

equivalent to an anti-CD20 antibody. A molecule that is functionally equivalent to an anti-CD20 antibody is a molecule which exhibits substantially the same binding activity as anti-CD20 antibodies known in the art. Such anti-CD20 antibodies that are known in the art include rituximab (Genentech/Biogen-IDEC), ocrelizumab (Genentech), PRO131921 (Genentech), GA101 (Roche), tositumomab (GlaxoSmithKline), ofatumumab (GenMab), veltuzumab (Immunomedics), AME-133 (Lilly), TRU-015 (Trubion), and the like. Although rituximab is used herein to exemplify the anti-CD20 antibody, other anti-CD20 antibodies known in the art or made using methods known in the art may be used in accordance with the present invention.

[54] As set forth in Table 1, there are at least 3 distinct classes of CpGs, differing in their specific nucleotide sequence and backbone structure, and their immunostimulatory activities. See Krieg AM (2007) J Clin Invest. 117:1184-1194; and Klinman DM (2004) Nat Rev Immunol. 4:249-258, which are herein incorporated by reference. Class A CpGs induce the strongest secretion of alpha-interferon by plasmacytoid DCs, but little B cell activation. In contrast, class B CpG induce strong B cell proliferation and differentiation, but only modest alpha-interferon secretion by plasmacytoid DCs. Class B CpGs include the major CpG products currently under development for cancer therapy, e.g. CpG 7909/PF-3512676 (Pfizer) and Dynavax ISS-1018). Class C CpGs, which possess unique structural features and combine immunomodulatory activities of both A and B-class CpGs, including strong alpha-interferon secretion and B cell activation.

Table 1
Classes of CpGs

ODN Type	Example	Structural Characteristics	Immunomodulatory Activity
Class A (D-type)	1585 (Mouse) GGggtcaac:ggggaGGGGGG (SEQ ID NO:1) 2336 (Human) GGGgacgac:gacgtgGGGGGG (SEQ ID NO:2)	Backbone contains both phosphodiester bases and phosphorothioate bases Single or multiple CpG motifs (bold) CpG flanking region forms a palindrome Poly-G tail at 3' end CpG motif imbedded in a palindrome	Induces exceptionally strong pDC IFN-alpha secretion and moderate expression of costimulatory molecules. Induces very little B cell activation. APC maturation, mediated by secreted IFN-alpha.
Class B (K-type)	1826 (Mouse) TCCATGACGTTCCTGACGTT (SEQ ID NO:3) 10103 (Human) TCGTCGTTTTCGGTCGTTTT (SEQ ID NO:4) 7909 (PF-3512676) (Human) TCGTCGTTTTGTCGTTTTGTCG TT (SEQ ID NO:5)	Fully modified phosphorothioate backbone Multiple CpG motifs 5' motif most stimulatory	Induces very strong B cell proliferation and differentiation, with IgM and IL-6 production. Induces pDC expression of costimulatory molecules and modest IFN-alpha secretion. pDC maturation and production of TNF.
Class C	2395 (Mouse/Human) TCGTCGTTTTCGGCGC:GCGCC G (SEQ ID NO:6)	Fully modified phosphorothioate backbone Multiple CpG motifs TCG dimer at 5' end CpG motif imbedded in a palindrome	Induces pDC IFN-alpha secretion and expression of costimulatory molecules. Induces strong B cell proliferation and differentiation with IgM and IL-6 production.

[55] Although the CpG exemplified herein is CpG 1826 (SEQ ID NO:3), other CpGs known in the art may be used in accordance with the present invention. See, for example, U.S. Patent Application Publication Nos. 20090082295, 20090087446, 20090117132, 20090017021, 20080226649, 20080045473, 20080009455, 20070232622, 20070224210, 20070142315, 20060211644, 20060188913, 20050059619, 20040198680, 20040152649, 20040092472, 20040067905, and 20040053880; and U.S. Patent Nos. 7,713,529, 7,674,777, 7,615,539, 7,605,138, 7,576,066, 7,569,553, 7,566,703, 7,524,828, 7,517,861, 7,488,490, 7,402,572, 7,271,156, 7,223,741, 6,949,520, 6,821,957, 6,653,292, 6,429,199, 6,406,705, 6,339,068, 6,239,116, 6,218,371, 6,207,646, 6,194,388, 7,381,807, 7,521,063, 6,562,798, 6,849,725, and 7,276,489; and the like, which are herein incorporated by reference.

[56] In some embodiments, the CpGs are class B CpGs. In some embodiments the CpGs are human CpGs. In some embodiments, the CpGs comprise at least 5 nucleotides. In some embodiments, the CpGs comprise 2 to 100, preferably about 8 to about 40, nucleotides. In some embodiments, the CpGs comprise 10 to 30 nucleotides. In some embodiments, the CpGs comprises 15-25 nucleotides. In some embodiments, the CpGs contain one or more the following structures: $[CGN_a]_x$, $[N_aCG]_x$, $[N_aCGN_b]_x$, $[N_aCGTTN_b]_x$, and $[N_aCGN_bCGN_c]_x$, where N is any nucleotide base, x is 0-25 and a, b and c are independently 1-15. For example, sequences which fall within $[N_aCGN_b]_x$ include ACGT, GTCGTT, TCGGTT, TGACGTT, and ACGTACGT. In some embodiments, the CpG is selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6.

[57] In some embodiments, the CpGs contain a GCG trinucleotide. In some embodiments, the CpGs are stabilized, particularly phosphorothioate stabilized. The CpGs may or may not be presence in a palindromic sequence.

[58] In some embodiments, the CpGs contain T-rich nucleic acids that contain poly T sequences and/or have greater than about 25% T nucleotide residues. Also encompassing TG nucleic acids having TG dinucleotides, and C-rich nucleic acids having at least one poly-C region and/or greater than about 50% C nucleotides.

[59] In some embodiments, the CpGs have a GTC trinucleotide in place of the CG dinucleotide. In some embodiments, the CpGs have one or more modified cytosines.

[60] In some embodiments, the CpGs are deoxyribonucleic acid molecules that are partially single-stranded, dumbbell-shaped, and covalently closed.

DEFINITIONS

[61] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

[62] As used herein, each of the following terms has the meaning associated with it in this section. The articles "a" and "an" are used herein to refer to one or to more than one (i.e., to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

[63] As used herein, a "CpG" is a nucleic acid molecule containing one or more cytosines followed by a guanosine and linked by a phosphate bond.

[64] As used herein "ODN" refers to an oligodeoxynucleotide sequence which may or may not be a CpG.

[65] As used herein, "control ODN" is used to refer to an oligodeoxynucleotide sequence which is not a CpG.

[66] As used herein, an "antibody-ODN conjugate" refers to an anti-CD20 antibody or control antibody conjugated to an ODN which may be a CpG or a control ODN. As provided herein, a specific antibody-ODN conjugate may be referred to by the names of the antibody and ODN, e.g. rituximab-CpG 1826 conjugate.

[67] As used herein, an "anti-CD20" antibody refers to an antibody or fragment thereof which specifically recognizes a CD20 antigen. Thus, the antiCD20 portion of an antiCD20-ODN conjugate according to the present invention is a molecule which specifically recognizes a CD20 antigen.

[68] As used herein, a "control conjugate" comprises control antibody, control ODN, or both.

[69] A "pharmaceutical composition" refers to a mixture of one or more of the compounds described herein, or physiologically/pharmaceutically acceptable salts, solvates, hydrates or prodrugs thereof, with other components, such as physiologically/pharmaceutically acceptable carriers and excipients. The purpose of a pharmaceutical composition is to facilitate administration of a compound to an organism.

[70] As used herein, a "physiologically/pharmaceutically acceptable carrier" refers to a carrier or diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound.

[71] A "pharmaceutically acceptable excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of a compound. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

[72] By the term "effective amount", or "therapeutically effective amount," as used herein, is meant an amount that when administered to a mammal, preferably a human, mediates a detectable therapeutic response compared to the response detected in the absence of the compound. A therapeutic response, such as, but not limited to, increased overall survival, inhibition of and/or decreased tumor growth (including tumor size stasis), tumor size, metastasis, and the like, can be readily assessed by a plethora of art-recognized methods, including, e.g., such methods as disclosed herein.

[73] The skilled artisan would understand that the effective amount of the antiCD20-CpG conjugate administered herein varies and can be readily determined based on a number of factors such as the disease or condition being treated, the stage of the disease, the age and health and physical condition of the mammal being treated, the severity of the disease, the particular antiCD20-CpG conjugate being administered, and the like.

[74] A "therapeutically effective amount", or "effective amount," is intended to qualify the amount of an agent required to detectably reduce to some extent one or more of the symptoms of a neoplasia disorder, including, but is not limited to: (1) reduction in the number of cancer cells; (2) reduction in tumor size; (3) inhibition (i.e., slowing to some extent, preferably stopping) of cancer cell infiltration into peripheral organs; (4) inhibition (i.e., slowing to some extent, preferably stopping) of tumor metastasis; (5) inhibition, to some extent, of tumor growth; (6) relieving or reducing to some extent one or more of the symptoms associated with the disorder; (7) relieving or reducing the side effects associated with the administration of anticancer agents; and/or (8) increasing, to some extent, the overall survival of a patient relative to that observed for the standard of care for a given tumor type or neoplastic disorder.

[75] A "maintenance effective amount" is intended to qualify the amount of an agent required to detectably maintain the therapeutic benefit achieved during a therapeutic regimen, including, but not limited to (1) inhibiting an increase in the number of cancer cells; (2) inhibiting an increase in tumor size; (3) inhibiting cancer cell infiltration into peripheral organs; (4) inhibiting tumor metastases; (5) relieving or reducing to some extent one or more of the symptoms associated with the disorder; and/or (6) inhibiting a recurrence or onset of one or more of the symptoms associated with the disorder.

[76] The therapeutically effective amount or maintenance effective amount of an antiCD20-CpG conjugate can be initially determined from animal models. The applied dose can be adjusted based on the relative bioavailability and potency of the administered antiCD20-CpG conjugate. Adjusting the dose to achieve maximal efficacy based on the methods described above and other methods as are well-known in the art is well within the capabilities of the ordinarily skilled artisan.

[77] Combined with the teachings provided herein, by choosing among the various active compounds and weighing factors such as potency, relative bioavailability, patient body weight, severity of adverse side-effects and preferred mode of administration, an effective prophylactic or therapeutic treatment regimen or maintenance phase can be planned which does not cause substantial toxicity and yet is entirely effective to treat the particular subject.

[78] As used herein, to "treat" means reducing the frequency with which symptoms of a disease (i.e., tumor growth and/or metastasis, or other effect mediated by the numbers and/or activity of immune cells, and the like) are experienced by a patient. The term includes the administration of the antiCD20-CpG conjugates of the present invention to prevent or delay the onset of the symptoms, complications, or biochemical indicia of a disease, alleviating the symptoms or arresting or inhibiting further development of the disease, condition, or disorder. Treatment may be prophylactic (to prevent or delay the onset of the disease, or to prevent the manifestation of clinical or subclinical symptoms thereof) or therapeutic suppression or alleviation of symptoms after the manifestation of the disease.

[79] As used herein, to "inhibit" means inhibiting the onset or development of symptoms of a disease (i.e., tumor growth and/or metastasis, or other effect mediated by the numbers and/or activity of immune cells, and the like) experienced by a subject. The term includes the administration of the antiCD20-CpG conjugates of the

present invention to inhibit or delay the onset of the symptoms, complications, or biochemical indicia of a disease.

[80] "Instructional material," as that term is used herein, includes a publication, a recording, a diagram, or any other medium of expression which can be used to communicate the usefulness of an antiCD20-CpG conjugate of the invention in a kit for affecting, alleviating or treating the various diseases or disorders recited herein. Optionally, or alternately, the instructional material can describe one or more methods of alleviating the diseases or disorders in a cell, a tissue, or a mammal, including as disclosed elsewhere herein.

[81] The instructional material of the kit may, for example, be affixed to a container that contains an antiCD20-CpG conjugate of the invention or be shipped together with a container that contains the compound and/or composition.

[82] Alternatively, the instructional material may be shipped separately from the container with the intention that the recipient uses the instructional material and the antiCD20-CpG conjugate cooperatively.

[83] The antiCD20-CpG conjugate of the invention may be provided in a medicinal dispenser. A medical dispenser is a package defining a plurality of medicinal storage compartments, each compartment for housing an individual unit of medicament. An entire medicinal course of treatment is housed in a plurality of medicinal storage compartments.

[84] A package defining a plurality of medicinal storage compartments may be any type of disposable pharmaceutical package or card that holds medicaments in individual compartments. For example, the package is a blister package constructed from a card, which may be made from stiff paper material, a blister sheet and backing sheet. Such cards are well known to those of ordinary skill in the art.

[85] As an example, a medicinal dispenser may house an entire medicinal course of treatment. The dispenser may include the day indicia to indicate which day the individual units of medicament are to be taken. These may be marked along a first side of the medicinal package. The dose indicia may also be marked, for example along a second side of the medicinal package perpendicular to the first side of the medicinal package, thereby indicating the time which the individual unit of medicament should be taken. The unit doses may be contained in the dispenser which is a blister pack.

[86] Except when noted, the terms "patient" or "subject" are used interchangeably and refer to mammals such as human patients and non-human primates, as well as veterinary subjects such as rabbits, rats, and mice, and other animals. Preferably, patient refers to a human.

[87] Unless otherwise defined herein, scientific and technical terms used in connection with the present invention shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. Generally, nomenclatures used in connection with, and techniques of, cell and tissue culture, molecular biology, immunology, microbiology, genetics and protein and nucleic acid chemistry and hybridization described herein are those well known and commonly used in the art.

[88] The methods and techniques of the present invention are generally performed according to methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification unless otherwise indicated. Such references include, e.g., Sambrook and Russell, Molecular Cloning, A Laboratory Approach, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (2001), Ausubel et al., Current Protocols in Molecular Biology, John Wiley & Sons, NY (2002), and Harlow and Lane Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1990), which are incorporated herein by reference. Enzymatic reactions and purification techniques are performed according to manufacturer's specifications, as commonly accomplished in the art or as described herein. The nomenclatures used in connection with, and the laboratory procedures and techniques of, analytical chemistry, synthetic organic chemistry, and medicinal and pharmaceutical chemistry described herein are those well known and commonly used in the art. Standard techniques are used for chemical syntheses, chemical analyses, pharmaceutical preparation, formulation, and delivery, and treatment of patients.

[89] Any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, as it will be understood that modifications and variations are encompassed within the spirit and scope of the instant disclosure.

[90] The following examples are intended to illustrate but not to limit the invention.

EXPERIMENTS

MICE AND TUMOR CELL LINES

[91] C3Hf/Sed/Kam mice (6-10 weeks old) were bred and housed in the Radiation Oncology Barrier Facility at the University of California, Los Angeles (UCLA), and experiments conducted according to UCLA guidelines. 38C13 (a gift from Ronald Levy, Stanford, CA) is a C3H B cell lymphoma expressing surface IgM,k. See Bergman et al. (1977) Eur J Immunol. 7:413-417, which is herein incorporated by reference. 38C13-huCD20 cells have been previously described. See Golay et al. (2006) Haematologica 91:176-183, which is herein incorporated by reference. The cell line 38C13-huCD20 IFNAR KD was produced by transducing 38C13-huCD20 cells with a lentiviral vector encoding GFP and an shRNA targeting the interferon alpha receptor (IFNAR)-1 subunit, flow sorted for high GFP expression, and stable transfectants cloned by limiting dilution.

[92] The A20 murine B cell lymphoma line was obtained from the American Type Culture Collection (ATCC) (Rockville, MD), transduced with a lentivirus encoding the human CD20 gene, stained with anti-human CD20-phycoerythrin (PE) or mouse IgG1,k-PE isotype control (BD Biosciences, San Jose, CA), and the highest-expressing cells collected by sterile flow cytometric sorting (FACS Vantage SE with Diva option, BD Biosciences), yielding the line A20-huCD20. See Golay et al. (2006) Haematologica 91:176-183, which is herein incorporated by reference. All tumor cells were cultured in RPMI 1640 medium (Invitrogen Corporation, Carlsbad, CA) supplemented with 10% heat-inactivated FCS (Omega Scientific, Tarzana, CA), 100 Units/ml penicillin/streptomycin, 2 mM L-glutamine, and 50 mM beta-mercaptoethanol (all from Invitrogen, RPMI complete media), at 37°C in 5% CO_2.

[93] Oligodeoxynucleotides (ODNs): CpGs and control ODNs

[94] As used herein "ODN" refers to an oligodeoxynucleotide sequence which may or may not be a CpG. As used herein, "control ODN" is used to refer to an oligodeoxynucleotide sequence which is not a CpG.

[95] Native, unmodified CpG 1826, 5'-TCCATGA**CG**TTCCTGA**CG**TT (SEQ ID NO:3; bold nucleotides represent the immunostimulatory CpG sequences), and control ODN 2138, 5'-TCCATGAGCTTCCTGAGCTT (SEQ ID NO:7), were purchased from Coley Pharmaceuticals Group (Wellesley, MA). Human CpG 10103,

5'- T**CG**T**CG**TTTTT**CG**GT**CG**TTTT (SEQ ID NO:4); bold nucleotides represent the immunostimulatory CpG sequences), and control ODN 2137, 5'-TGCTGCTTTTGTGCTTTTGTGCTT (SEQ ID NO:8), were purchased from InvivoGen (San Diego, CA). 5'-amino-labeled CpG 1826, control ODN 2137, and control ODN 2138 were purchased from Solulink (San Diego, CA).

[96] Antibody-CpG Conjugation

[97] The antibodies exemplified in the experiments herein are rituximab (anti-CD20 antibody) and trastuzumab (control antibody, i.e. not an anti-CD20 antibody) which were obtained from Genentech, Inc. (South San Francisco, CA). As used herein, an "antibody-ODN conjugate" refers to an anti-CD20 antibody or control antibody conjugated to an ODN which may be a CpG or a control ODN. As provided herein, a specific antibody-ODN conjugate may be referred to by the names of the antibody and ODN, e.g. rituximab-CpG 1826 conjugate.

[98] Two methods known in the art were adapted for the purpose of generating the cleavable, antibody hinge region-linked ODN conjugates exemplified herein. The first method is based on that described in Betting et al. (2008) J. Immunol. 181: 4131-4140; and WO 2007106435, which are herein incorporated by reference. The second method is based on that described in Bailey et al. (2007) J. Amer. Chem. Soc. 129:1959-1967, which is herein incorporated by reference.

[99] METHOD 1 - SMCC: Figure 11 depicts the use of sulfo-SMCC to conjugate CpG to antibody via a cleavable linker.

[100] Antibodies were first partially reduced using 0.1 mM dithiothrietol (DTT, Sigma-Aldrich) for 1 hour at 37°C, then dialyzed into 1X phosphate buffered saline (PBS) containing 0.1 M EDTA, pH 6.5 to prevent re-oxidation of sulfhydryl groups. 5'-amino-labeled ODN was exchanged into carbonate buffer pH 8.5 using VIVASPIN 500 spin filter units (Sartorius Mechatronics, Bohemia, NY), concentration determined by A_{260} spectrophotometry, and incubated with 50-fold molar excess sulfosuccinimidyl-4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (sulfo-SMCC, Pierce, Rockford, IL) per manufacturer's instructions in carbonate buffer for 30 minutes at room temperature. After centrifugation to remove any insoluble SMCC, the SMCC-activated ODNs were exchanged into PBS with 0.1 M EDTA, pH 6.5, and incubated with the reduced antibody (1:5 molar ratio modified ODN:antibody) for about 4-8 hours at room temperature, allowing formation of the antibody-ODN

conjugates. The conjugates were dialyzed into PBS and purified by fast protein liquid chromatography (FPLC) using an ÄKTApurifier™ with GE Superdex 200 Size exclusion column (GE Healthcare Bio-Sciences, Piscataway, NJ). The final protein concentrations were determined using the BCA method (Pierce, Rockford, IL) and final oligonucleotide concentration by A_{260} spectrophotometry.

[101] Where SMCC precedes a conjugate, e.g. SMCC rituximab-CpG 1826, the conjugate was formed according to this method. SMCC rituximab-CpG conjugates retained the ability of rituximab to bind to human CD20+ lymphoma cells. SMCC rituximab-CpG 1826 retained the biologic activity of free CpG 1826 to upregulate the costimulatory molecule ICAM-1/CD54 on the surface of B cell lymphoma cells. Specifically, as shown in Figure 12, human CD20-expressing lymphoma cells incubated with equivalent concentrations of free CpG 1826 or SMCC rituximab-CpG 1826 show identical levels of ICAM-1/CD54 upregulation. Thus, the biological activity of a CpG is retained when coupled to an antibody.

[102] METHOD 2 - MHPH/4FB: To improve the yield of antibody-ODN conjugates, bis-arylhydrazone chemistry, which offers improved conjugation efficiency and traceable intermediates during the conjugation process, was employed. Four mg of 5'-amino-labeled ODN was exchanged into 1X 4FB modification buffer (0.1 M PBS, pH 7.2, 0.15 M NaCl, 0.05% sodium azide) using VIVASPIN 500 spin filter units, concentration determined by A_{260} spectrophotometry, and ODN concentration yielding 0.7 OD 260/µl in 150 µl final volume. C6-Succinimidyl 4-formylbenzoate (S-SS-4FB, Solulink, Inc., San Diego, CA) dissolved in dimethylformamide (DMF) at 100 mg/ml. The quantity of S-SS-4FB to be reacted with the ODN was determined using the amino oligo modification calculator supplied by Solulink (See hypertext transfer protocol:// world wide web.solulink.com/protocols.php (wherein hypertext transfer protocol = http, world wide web = www), using the 1826 molar extinction coefficient of 181100. The entire volume of 5'-amino-labeled ODN was mixed with 20 equivalents (47.6 µl) of S-SS-4FB linker, plus an additional 75 µl of DMF, and incubated for 2 hours at room temperature. After centrifugation to remove any insoluble materials, the modified ODN in the supernatant was exchanged into 1X conjugation buffer (0.1 M PBS, pH 7.2, 0.15 M NaCl, 0.05% sodium azide) using VIVASPIN 500 spin filter units, and the final concentration adjusted to about 0.3-0.5 OD 260/µl.

[103] To quantify the 4FB modification level of the ODN, the molar substitution ratio of the modified ODN was determined according to the manufacturer's instructions. Antibody solution at 10 mg/ml was exchanged into PBS with 0.1 M EDTA, pH 7.2 using Zeba Spin desalting columns (Pierce, Rockford, IL). To a 6 mg vial of 2-mercaptoethylamine HCl (2-MEA, Pierce, Rockford, IL), 1 ml of antibody solution was added, resulting in a final 2-MEA concentration of 50 mM, and the reaction mixture incubated for 90 minutes at 37°C to partially reduce the antibody. After cooling, the 2-MEA was separated from the reduced IgG using a Zeba desalt spin column with exchange into 1X MHPH modification buffer (0.1 M Phosphate Buffer, pH 7.2, 0.15 M NaCl, 0.05 M EDTA, 0.05% Azide). Ellman's reagent was used to confirm successful antibody reduction (Pierce, Rockford, IL). One mg of 3-N-Maleimido-6-hydraziniumpyridine hydrochloride (MHPH) was dissolved into 100 ml of DMF. 80.2 ml of linker was added to 20 mg of antibody according to the Solulink protein modification calculator (hypertext transfer protocol:// world wide web.solulink.com/protocols.php, wherein hypertext transfer protocol = http, world wide web = www), setting the equivalents of linker value to 25. This antibody-linker mixture was incubated for 2 hours at room temperature. After buffer exchange into 1X conjugation buffer using Zeba Spin desalt columns, the protein concentration was determined by BCA, and the MHPH-antibody linkage verified using the HyNic molar substitution ratio (MSR) determination protocol (Solulink, Inc., San Diego, CA).

[104] To complete the conjugation, 4FB modified ODN and MHPH-antibody were mixed together and incubated for about 4-8 hours at room temperature, in concentrations determined by the protein-oligonucleotide conjugation calculator (Solulink, Inc., San Diego, CA). The conjugates were purified by buffer exchange into PBS using VIVASPIN 15 columns with MW cutoff of 30KDa, then FPLC using the ÄKTApurifier™ with GE Superdex 200 Size exclusion column and collection of fractions expressing an overlapping A_{260}, A_{280}, and A_{354} signal. Concentrated fractions were pooled and the purified conjugates were quantitated using BCA to determine protein content and A_{260} for DNA content. Stoichiometry calculations indicated that conjugates contained an average of about 4 ODN molecules per antibody molecule.

[105] Where MHPH/4FB precedes a conjugate, e.g. MHPH/4FB rituximab-CpG conjugate, the conjugate was formed according to this method. Figure 13 illustrates MHPH/4FB conjugation chemistry. MHPH/4FB antibody-CpG conjugates provides

two potential cleavage sites for releasing the CpG from the antibody; one within the sulfhydryl-MHPH linkage, and another within the disulfide bond adjacent to the 4FB moiety.

[106] Tumor Cell and Dendritic Cell Activation Studies

[107] A20-huCD20 cells were cultured for 48 hours in RPMI complete media alone, or media containing 10 mg/ml free CpG 1826 or rituximab-CpG 1826 conjugate (with 10 mg/ml CpG content). Cells were harvested and stained with FITC-labeled antibody against intercellular adhesion molecule-1 (ICAM-1/CD54) or isotype control antibody (BD Biosciences) and analyzed by flow cytometry. Bone marrow derived dendritic cells were prepared as previously described (See Franki et al. (2008) Blood 111:1504-1511, which is herein incorporated by reference), harvested on day 6, and cultured overnight with media alone, 10 μg/ml free CpG 1826, rituximab, or MHPH/4FB rituximab-CpG 1826 conjugate (with 10 μg/ml CpG content). Non-adherent cells were then harvested, stained with FITC-labeled antibodies against CD80/B7.1, CD86/B7.2, MHC class II, or isotype control antibodies (BD Biosciences), and analyzed by flow cytometry using a BD FACSCalibur flow cytometer (BD Biosciences) with FACS Express software (De Novo Software, Los Angeles, CA).

[108] Figure 14 shows the activation of dendritic cells by the antiCD20-CpG conjugates of the present invention.

[109] Animal Therapy Experiments

[110] Mice were injected s.c. on the hind flank with 5 x 10^3 38C13-huCD20 tumor cells in Hank's buffered saline solution (HBSS) on day 0. After tumor establishment for 5 or 7 days, mice were treated intravenously (i.v.) with HBSS, 50 mg free CpG 1826, rituximab-CpG 1826 conjugate, a control conjugate, or a mixture of free CpG 1826 and rituximab. As used herein, a "control conjugate" comprises control antibody, control ODN, or both. ODN doses were always 50 mg. Conjugate doses were chosen to contain 50 mg of ODN, and identical doses of unconjugated antibodies served as controls. For comparing intratumoral injection of free CpGs to antiCD20-CpG conjugates of the present invention, 50 mg of free CpG 1826 was delivered i.t. by injection into the site of tumor inoculation on days 7 and 10. Animals not cured of their tumors were sacrificed when tumors reached 1.4 cm in diameter per

institutional guidelines. Survival differences among groups of mice were assessed using the Kaplan-Meier method with the log-rank test using Prism software (Graph-Pad Software, San Diego, CA). P values were considered statistically significant at $P < 0.05$.

[111] *In Vivo* Depletion Experiments

[112] Depletions of NK cells, macrophages and T cells were performed as previously described. See Golay et al. (2006) Haematologica 91:176-183, which is herein incorporated by reference. NK cells were depleted by injecting 50 μl of asialo-GM1 i.p. on days 0, 5, and 10. Complement depletions were achieved by treating mice with 25 U of cobra venom factor (Quidel Corporation, San Diego, CA) on days 0, 3, 6, and 9. Clodronate for preparing clodronate liposomes was a kind gift from Roche Diagnostics (Mannheim, Germany) and liposomes were prepared as previously described. Macrophage depletions were performed by injecting 150 μl of clodronate encapsulated liposomes i.p. on days 0, 3, 6, 9, and 12 starting on the day of tumor challenge. PBS-containing liposomes were injected as a control. $CD4^+$ and $CD8^+$ T cell subsets were depleted as previously described.

[113] Treatment of Type I Interferon-Resistant Tumors

[114] The cell line 38C13-huCD20 IFNAR KD was produced by transducing 38C13-huCD20 cells with a lentiviral vector encoding GFP and an shRNA targeting the interferon alpha receptor (IFNAR)-1 subunit, flow sorted for high GFP expression, and stable transfectants cloned by limiting dilution. Groups of 8 mice were inoculated on day 0 with either 38C13-huCD20 or 38C13-huCD20 IFNAR KD, and treated as indicated on days 7 and 10 with either HBSS or rituximab-CpG 1826 conjugate.

[115] Human Lymphoma Cell Proliferation Studies

[116] Human non-Hodgkin B cell lymphoma cells were cultured overnight in media alone, or in the presence of varying concentrations of free CpGs or control ODNs as indicated. Proliferation was measured by scintillation counting after an 8 hour pulse with ^{3}H-thymidine.

[117] To the extent necessary to understand or complete the disclosure of the present invention, all publications, patents, and patent applications mentioned herein are expressly incorporated by reference therein to the same extent as though each were individually so incorporated.

[118] Having thus described exemplary embodiments of the present invention, it should be noted by those skilled in the art that the within disclosures are exemplary only and that various other alternatives, adaptations, and modifications may be made within the scope of the present invention. Accordingly, the present invention is not limited to the specific embodiments as illustrated herein, but is only limited by the following claims.

We claim:

1. A method for treating or inhibiting a B cell malignancy in a subject which comprises administering to the subject the antiCD20-CpG conjugate according to any one of claims 5 to 12.

2. The method of claim 1, wherein the B cell malignancy is a CD20-positive B cell malignancy.

3. The method of claim 1, wherein the B cell malignancy is a B cell lymphoma.

4. The method according to any one of claims 1 to 3, wherein the antiCD20-CpG conjugate is systemically administered to the subject.

5. An antiCD20-CpG conjugate which comprises an antiCD20 portion linked with a linker to a CpG molecule.

6. The antiCD20-CpG conjugate of claim 5, wherein the linker is a cleavable linker.

7. The antiCD20-CpG conjugate of claim 5, wherein the antiCD20 portion is an anti-CD20 antibody or a molecule which specifically binds CD20.

8. The antiCD20-CpG conjugate of claim 5, wherein the CpG molecule is a class B CpG.

9. The antiCD20-CpG conjugate of claim 5, wherein the CpG molecule is CpG 1826, CpG 10103, or CpG 7909.

10. The antiCD20-CpG conjugate of claim 5, wherein the CpG molecule comprises one or more of the following sequences selected from the group consisting of $[CGN_a]_x$, $[N_aCG]_x$, $[N_aCGN_b]_x$, $[N_aCGTTN_b]_x$, and $[N_aCGN_bCGN_c]_x$, where N is any nucleotide base, x is 0-25 and a, b and c are independently 1-15.

11. The antiCD20-CpG conjugate according to any one of claims 5 to 10, wherein the antibody is rituximab, ocrelizumab, PRO131921, GA101, tositumomab, ofatumumab, veltuzumab, AME-133), or TRU-015.

12. The antiCD20-CpG conjugate of claim 5, wherein the antiCD20-CpG conjugate is rituximab-CpG 1826, rituximab-CpG 10103, or rituximab-CpG 7909.

13. The invention as described herein.

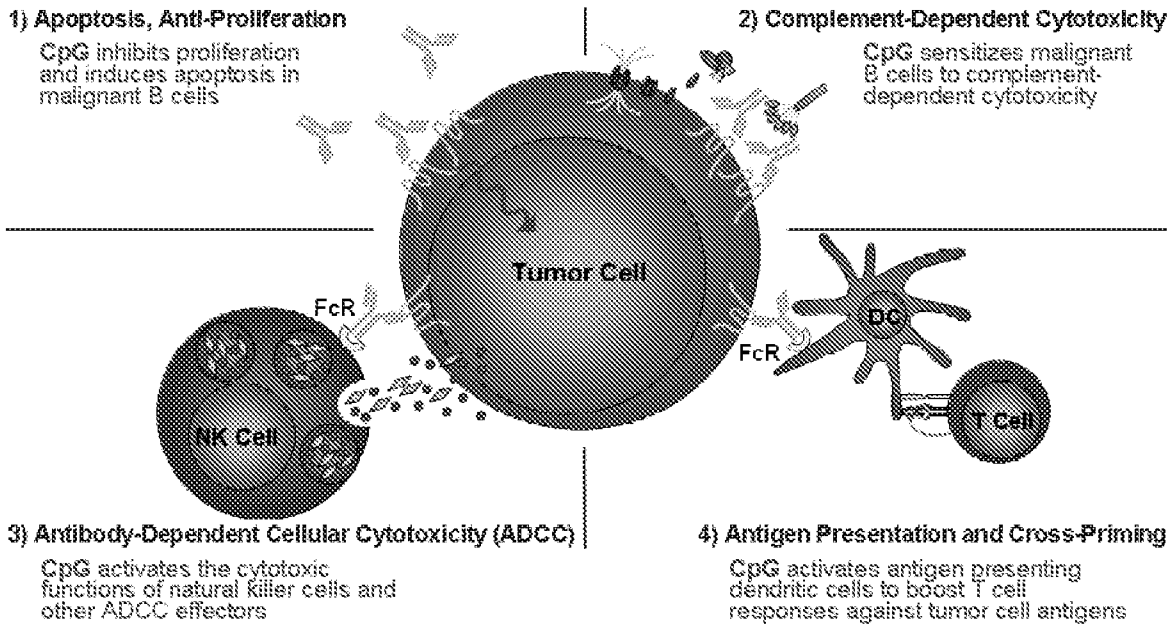


Figure 1

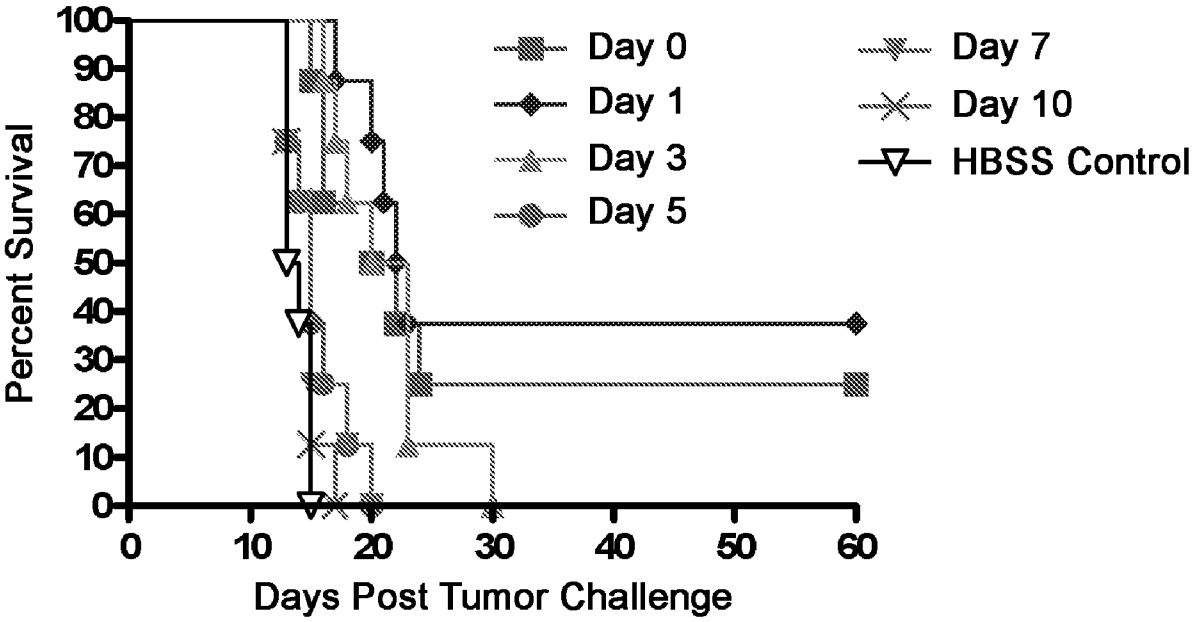


Figure 2

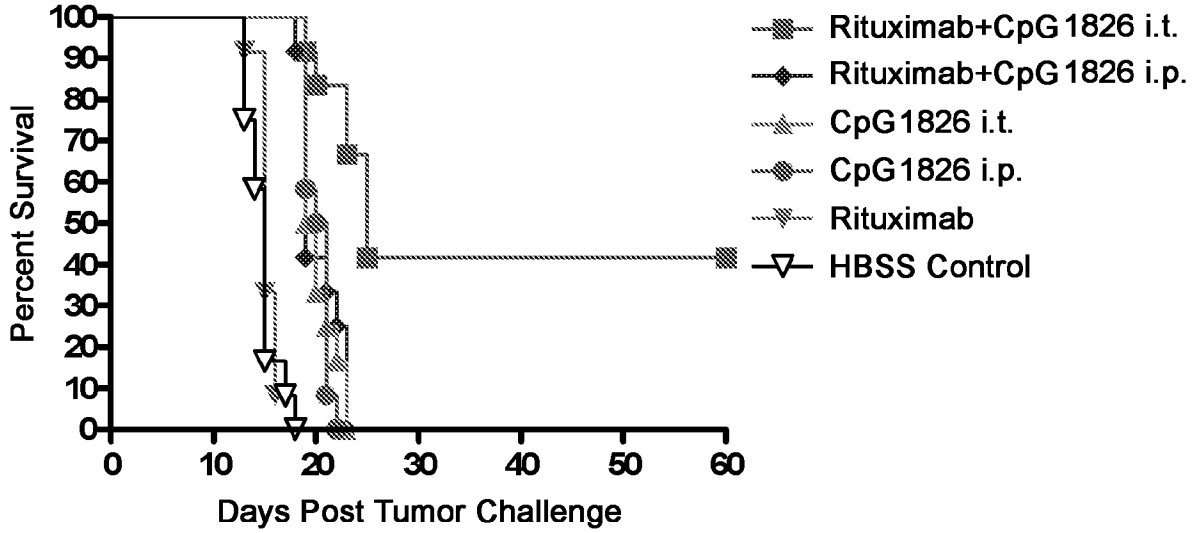


Figure 3A

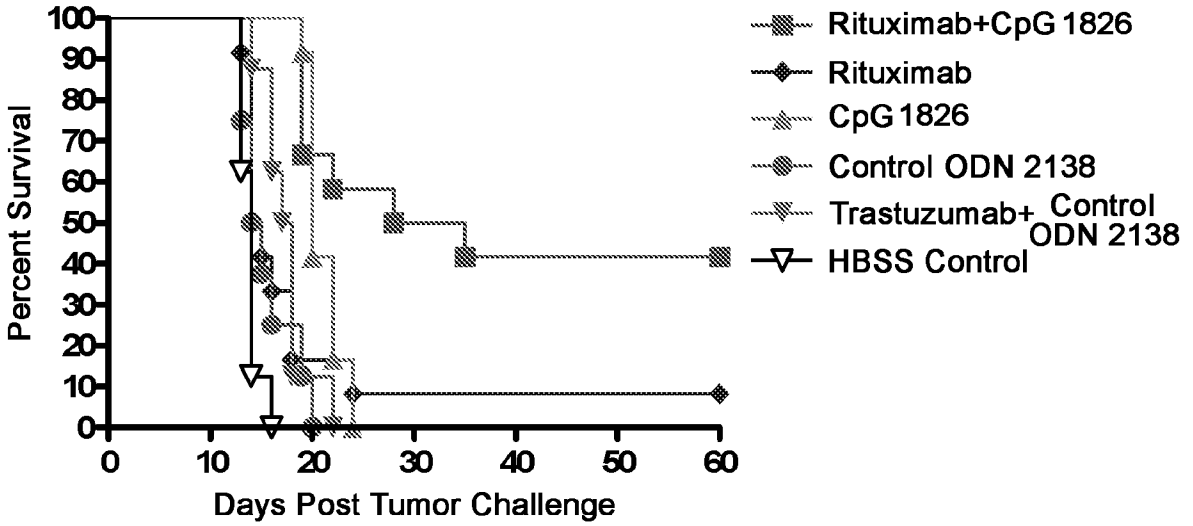
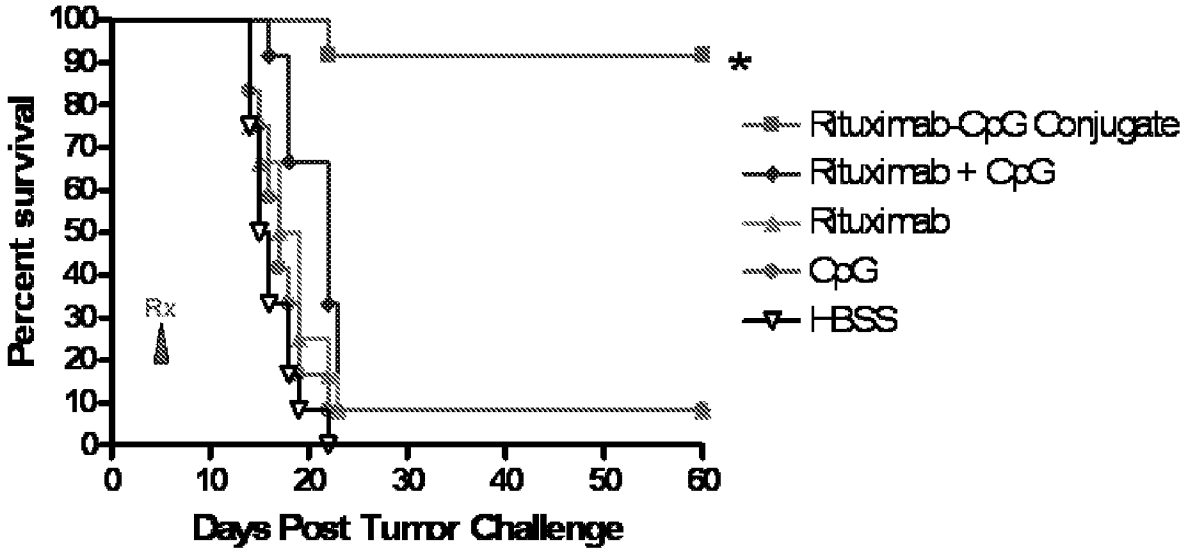


Figure 3B



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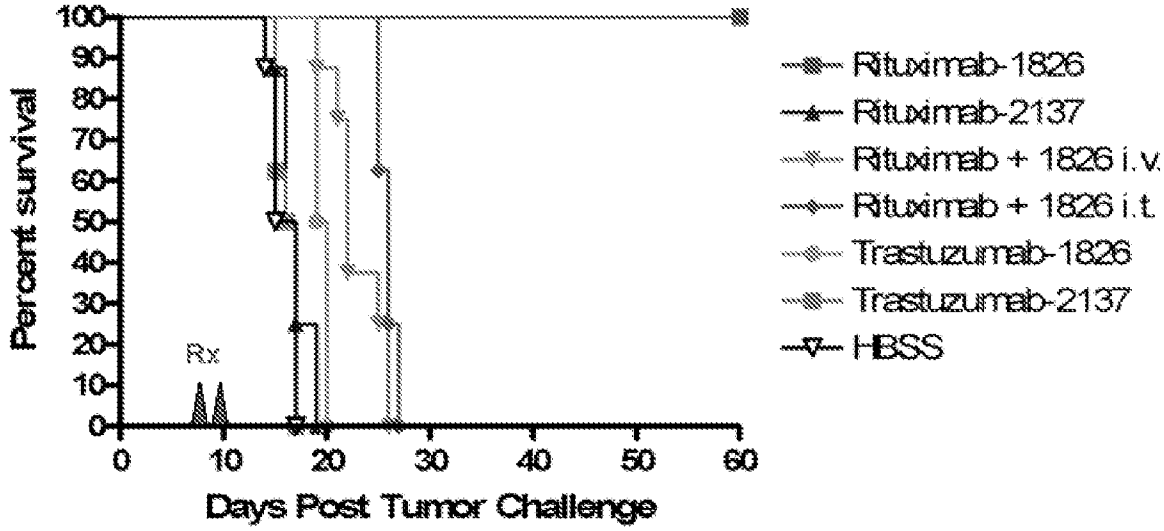

Figure 5

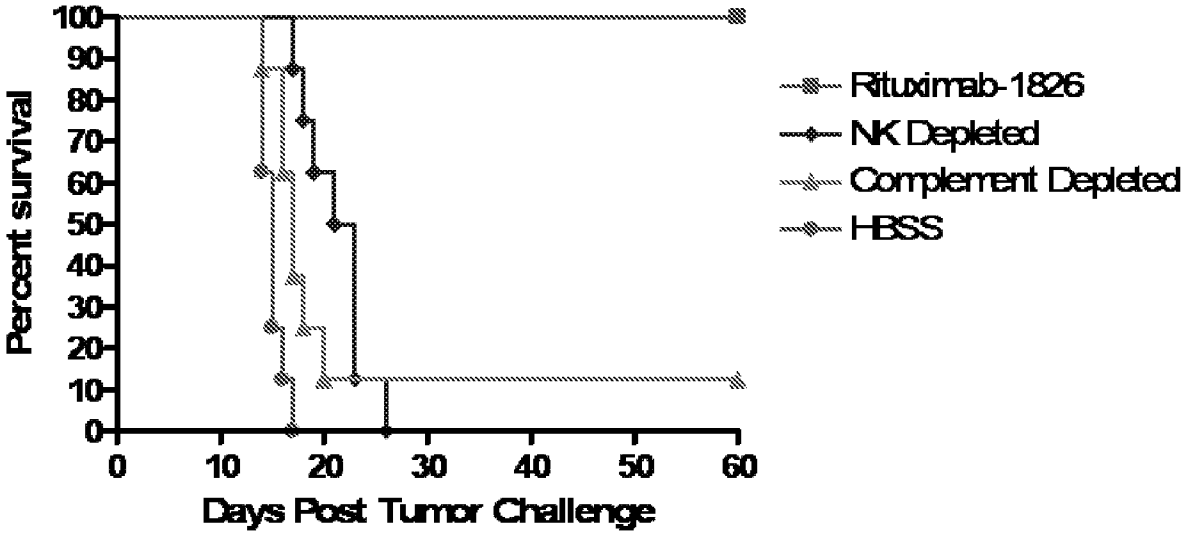

Figure 6

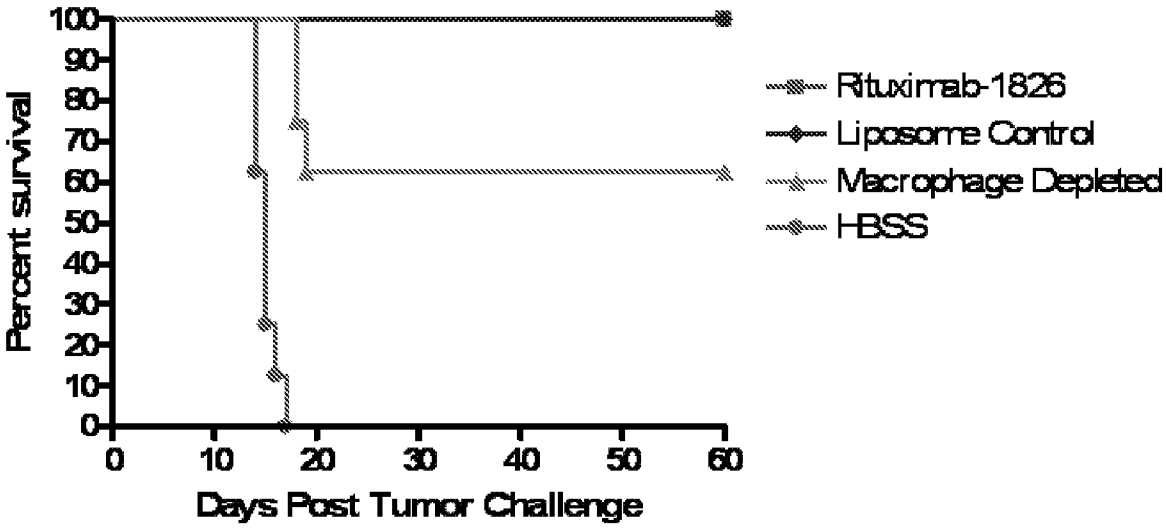

Figure 7

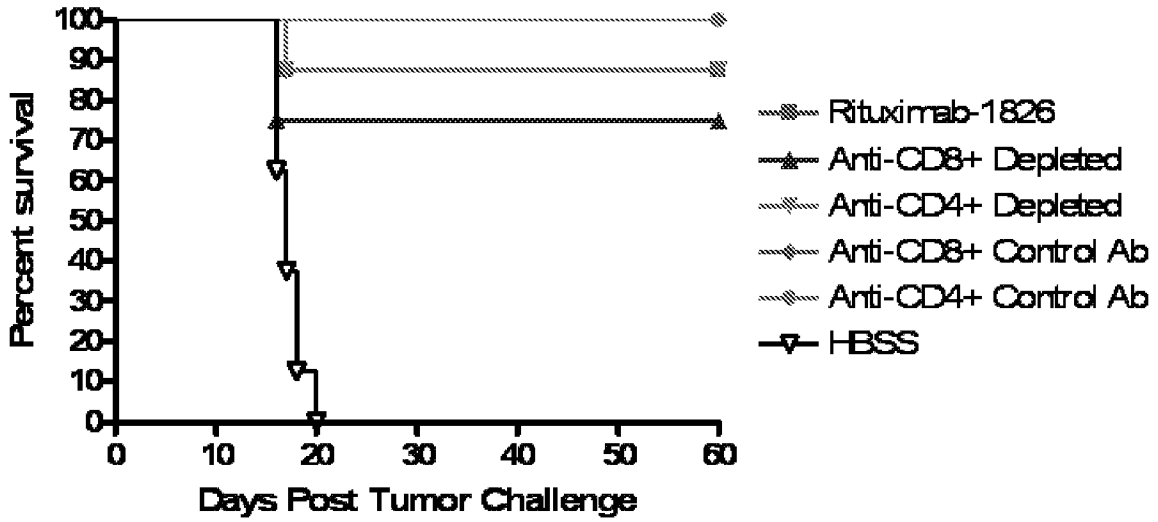

Figure 8

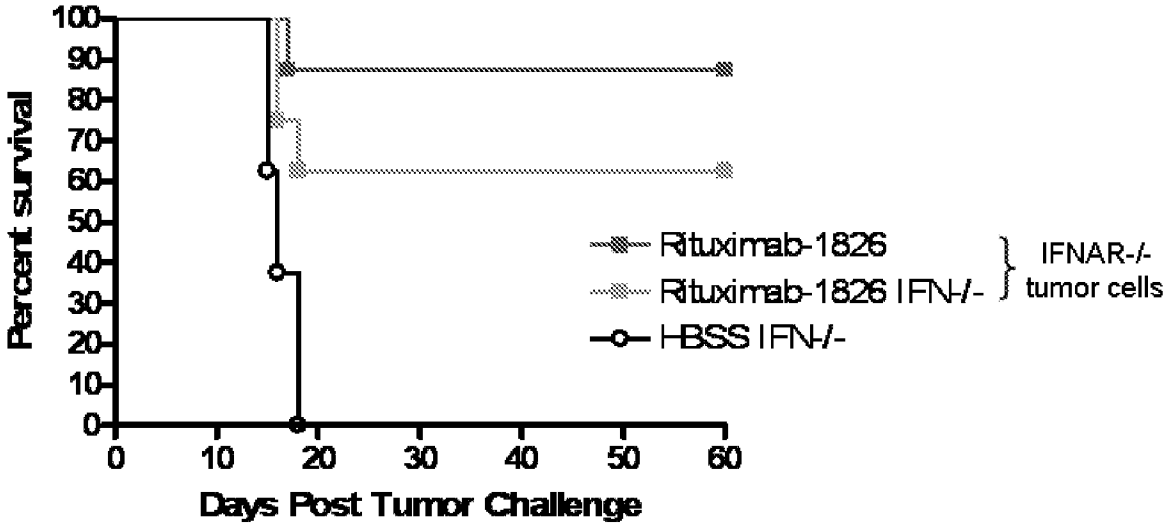

Figure 9

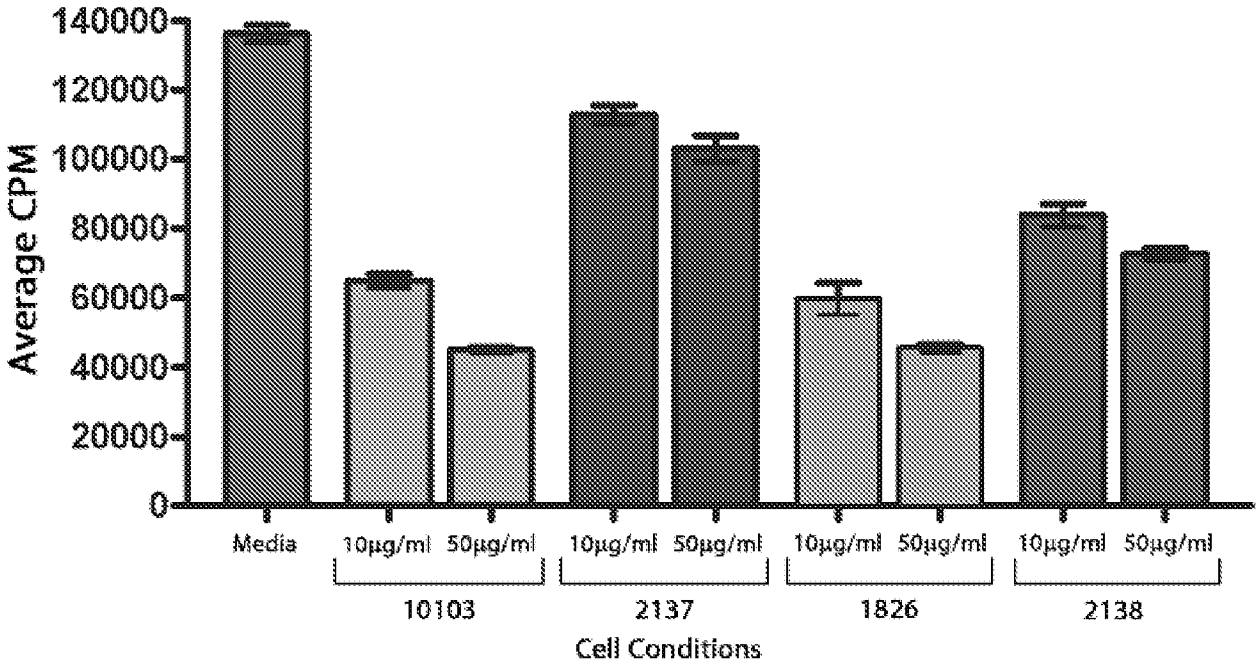

Figure 10A

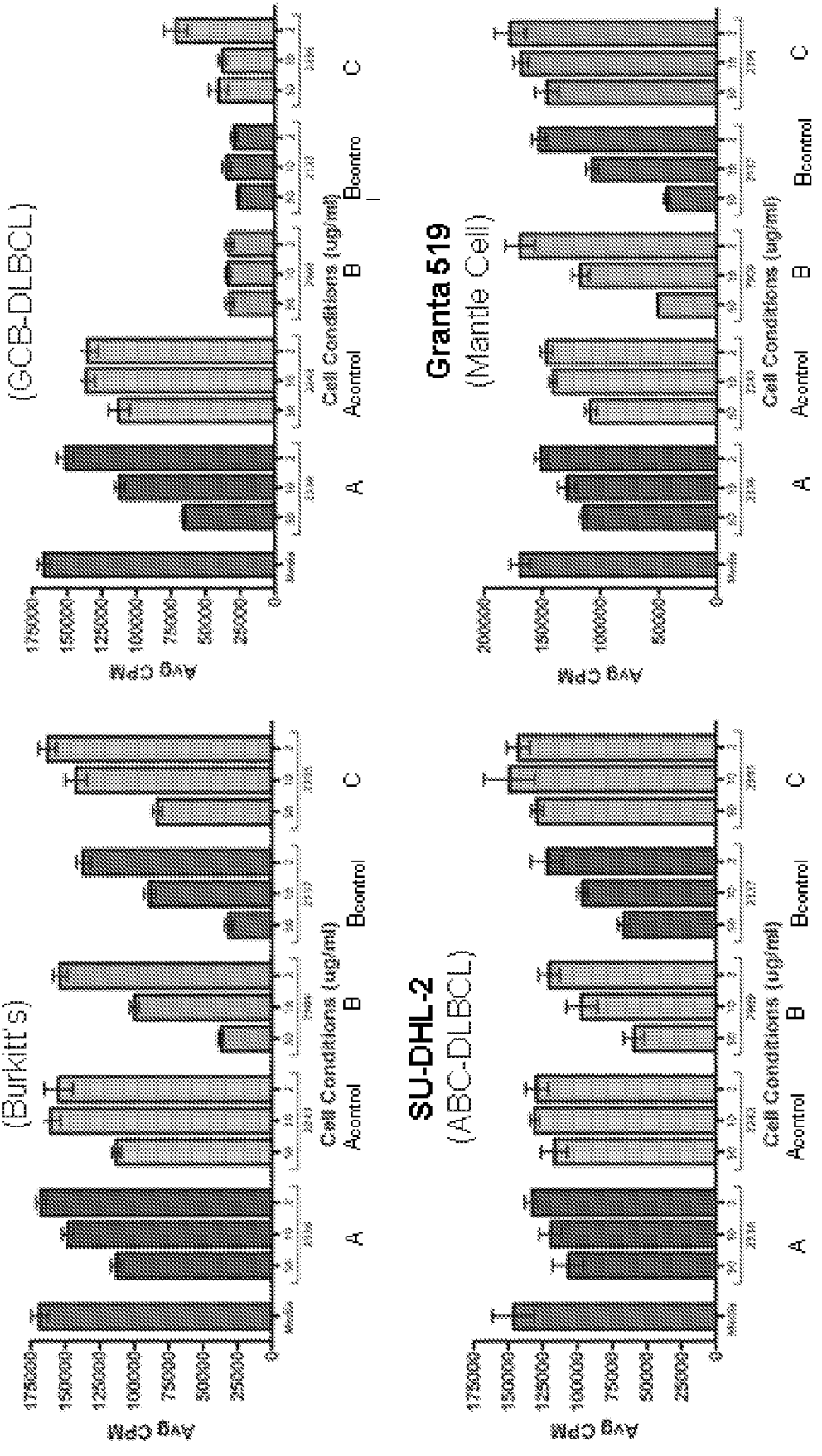
Raji
(Burkitt's)
OCI-Ly19
(GCB-DLBCL)
SU-DHL-2
(ABC-DLBCL)
Granta 519
(Mantle Cell)
Avg CPM
Cell Conditions (ug/ml)
Acontrol
B
Bcontrol
C
A

Figure 10B

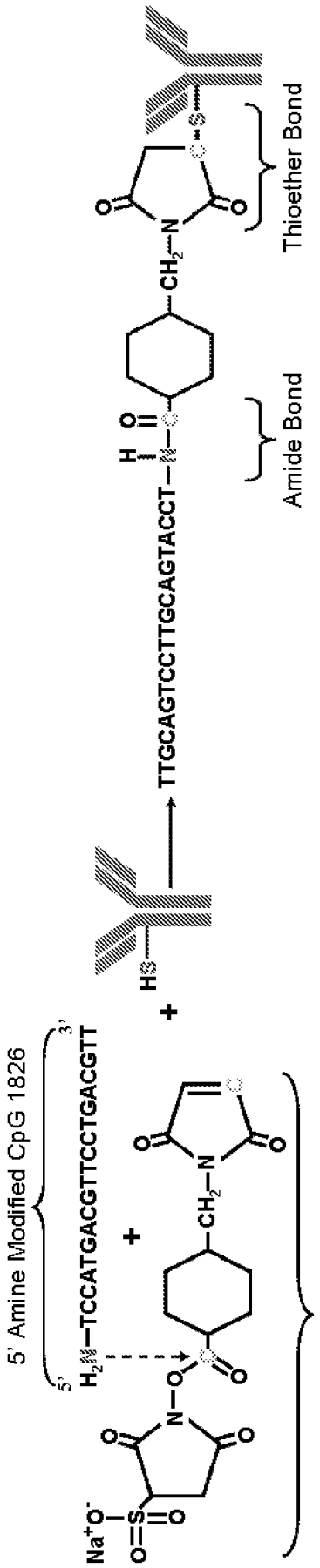
5' Amine Modified CpG 1826
5'
3'
H2N—TCCATGACGTTCCTGACGTT
Sulfo-SMCC
HS
TTGCAGTCCTTGCAGTACCT
Amide Bond
Thioether Bond

Figure 11

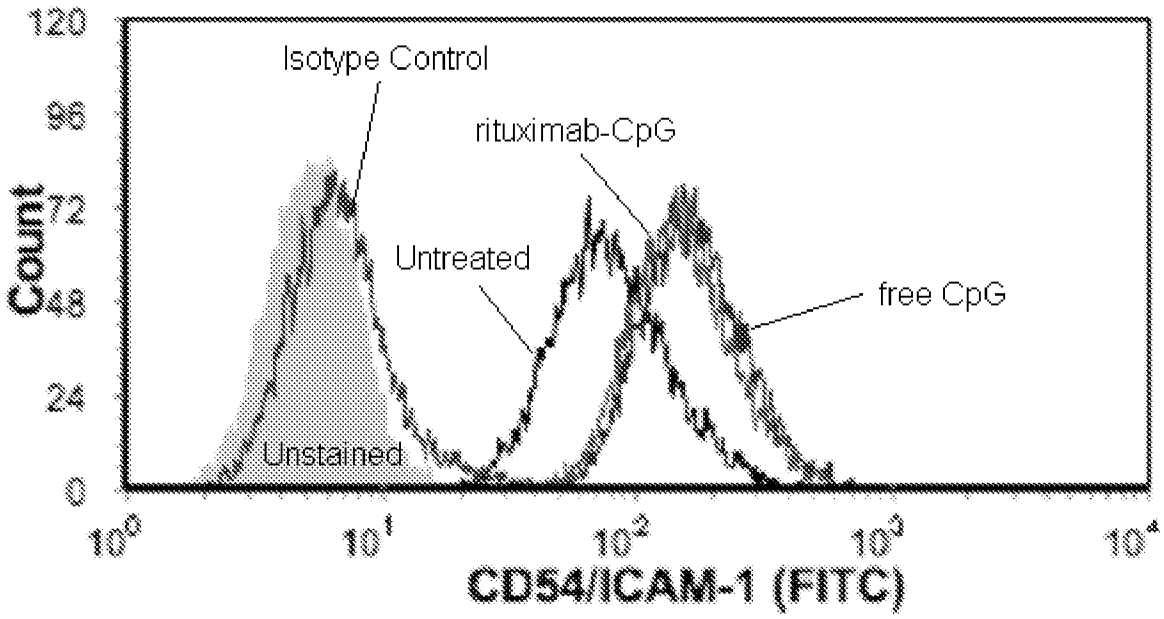
120
96
72
48
24
0
Count
Isotype Control
rituximab-CpG
Untreated
free CpG
Unstained
10^0
10^1
10^2
10^3
10^4
CD54/ICAM-1 (FITC)

Figure 12

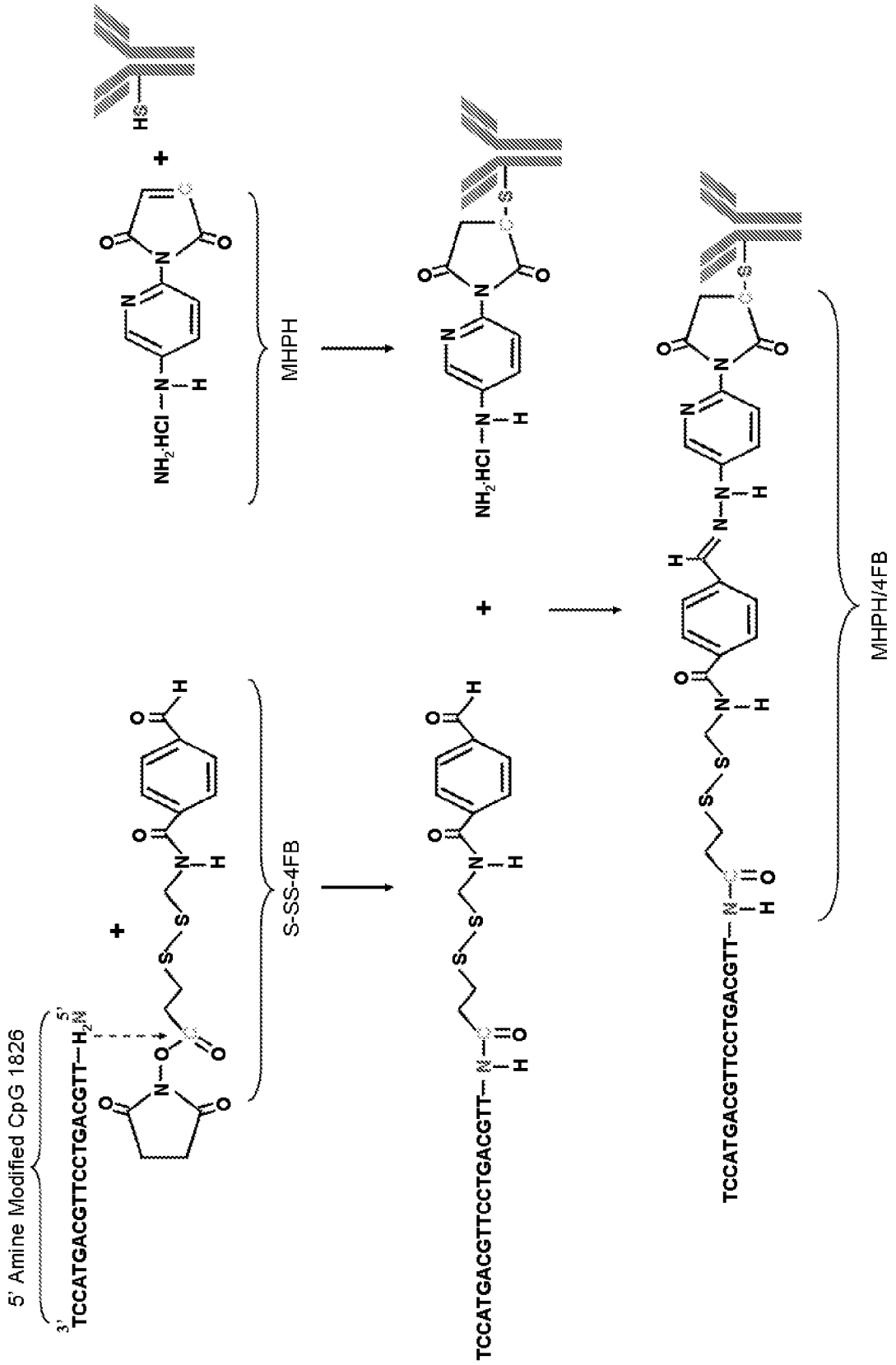
5' Amine Modified CpG 1826
3' TCCATGACGTTCCTGACGTT—H2N 5'
S-SS-4FB
MHPH
TCCATGACGTTCCTGACGTT
TCCATGACGTTCCTGACGTT
MHPH/4FB

Figure 13

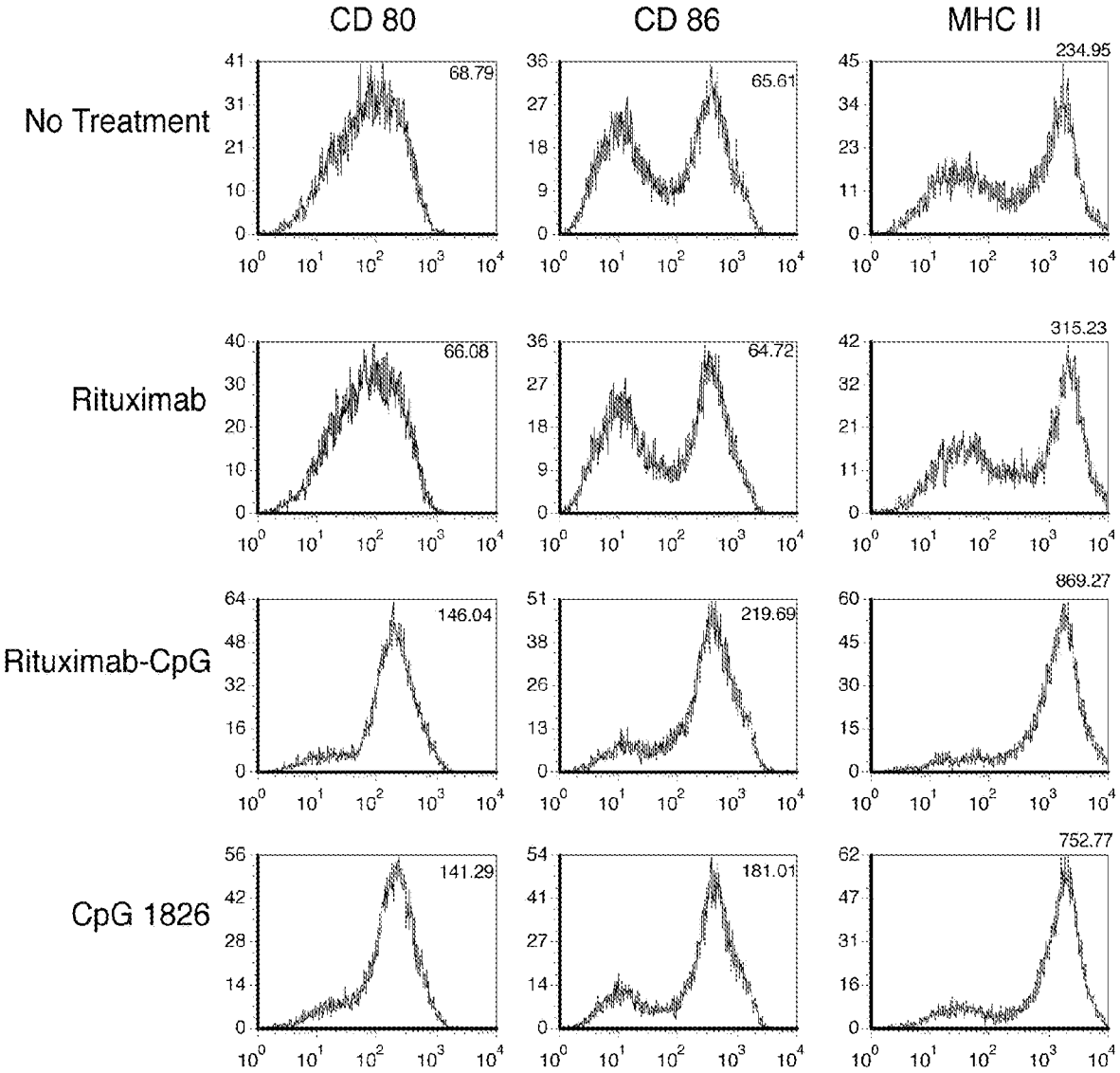
CD 80
CD 86
MHC II
No Treatment
Rituximab
Rituximab-CpG
CpG 1826
68.79
65.61
234.95
66.08
64.72
315.23
146.04
219.69
869.27
141.29
181.01
752.77

Figure 14