Title
Characterization of anti-CD20-TLR9 agonist conjugates in a syngeneic immunocompetent model for B cell lymphoma

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A thesis submitted in partial satisfaction
of the requirements for the degree Master of Science
in Microbiology, Immunology, and Molecular Genetics

by

Jenny L Park

2013
ABSTRACT OF THE THESIS

Characterization of anti-CD20-TLR9 agonist conjugates
in a syngeneic immunocompetent model for B cell lymphoma

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Jenny L Park

Master of Science in Microbiology, Immunology, and Molecular Genetics
University of California, Los Angeles, 2013
Professor Sherie Morrison, Chair

Anti-CD20 monoclonal antibodies (mAbs) have revolutionized the therapy of B cell lymphomas, but are only partially effective. While current genetic engineering and efforts are yielding mAbs with enhanced antibody-dependent cellular functions, these improvements are largely incremental and do not result in altering the tumor microenvironment and host immune cells to favor immune-mediated tumor destruction. We hypothesize that the efficacy of anti-lymphoma mAbs can be improved by linking these precise tumor-targeting vehicles to immunomodulatory substances for release within the tumor microenvironment. Toll-like receptor 9 (TLR9) agonist CpG oligodeoxynucleotides (ODN) are prime candidates for boosting anti-lymphoma immunity. Thus we aimed to characterize the activity and mechanisms of action of anti-CD20-CpG conjugates in a syngeneic immunocompetent model for B cell lymphomas.

We confirmed that anti-CD20-CpG conjugates can be purified from unconjugated antibody and CpG while maintaining biological activity. Confocal microscopy studies with
fluorescently-labeled conjugates revealed cellular uptake on human CD20 expressing mouse lymphoma cells, suggesting CpG is able to enter the cell even when targeting a non-internalizing target. Lastly, conjugates had superior normal B cell depletion efficiency compared to rituximab; even in more resistant B cell compartments such as the peritoneal cavity.
The thesis of Jenny L Park is approved.

John M. Timmerman
Genhong Cheng
Manuel Penichet
Sherie Morrison, Chair

University of California, Los Angeles

2013
DEDICATIONS

I dedicate this work to my mother, who ironically recently battled diffuse large B cell lymphoma. She is the strongest woman I know, and it is her strength and tenacity that drives me everyday.

I also dedicate this work and give special thanks to Dr. Timmerman, who touched my life as a motivational graduate student mentor, a compassionate doctor, and an unforgettably supportive figure.
TABLE OF CONTENTS

Abstract ...........................................................................................................................................ii-iii
Committee Page ................................................................................................................................iv
Dedications Page .............................................................................................................................v
Introduction .......................................................................................................................................1-5
  B cell non-Hodkin’s lymphoma ..............................................................................................1
  Monoclonal antibody therapy: Rituximab ...............................................................................1
  Improving monoclonal antibody therapy efficacy ...............................................................3-4
  Toll-like receptor 9 agonist as immunostimulants ..............................................................4-5
Study Rationale ...............................................................................................................................5-6
  Intratumoral delivery of CpG has potent activity against lymphomas ..............................5
  Anti-CD20-CpG conjugates target TLR9 agonist activity directly to tumor sites in vivo ...6
  Syngeneic, immunocompetent tumor models are required for preclinical evaluation of anti-CD20-CpG conjugates .....................................................................................................................6-7
Specific Aim 1 ..................................................................................................................................7-15
  Generate and characterize anti-CD20-CpG conjugates with respect to stoichiometry and biological activity ..........................................................................................................................7-15
Specific Aim 2 ..................................................................................................................................15-19
Specific Aim 3 ..................................................................................................................................19-22
  Evaluate the efficacy of anti-CD20-CpG conjugates in depleting the normal B cell compartment in huCD20 transgenic mice .................................................................................................19-22
Summary & Future Directions ..........................................................................................................22-23
References .........................................................................................................................................24-28
INTRODUCTION

B cell non-Hodgkin lymphoma

Non-Hodgkin lymphoma (NHL) is the sixth most common cancer in females and seventh in males in the US with an estimated 69,740 new cases and 19,020 deaths in 2013 (1). NHL encompasses a heterogeneous group of cancers, 85-95% of which arise from B cell that can be further categorized into indolent (low-grade) and aggressive (high-grade) histologic subtypes (2). Indolent NHLs (i.e. follicular lymphoma) are characterized by their slow growth and long natural history while aggressive NHLs (i.e. diffuse large B cell lymphoma) have a higher proliferative rate (3, 4).

Various treatment options are currently available, making B cell NHLs a targetable and even curable cancer. The B cell-specific 33-35 kDa transmembrane protein CD20 is stably expressed on over 90% of B cell NHLs (5). CD20 is expressed across most of the committed stages of normal B cell development, from pre-B cell to mature, activated, and memory B cells, but not on terminally differentiated plasma cells. The biological function of CD20 is unclear, although some evidence suggests a role in B cell differentiation, activation, and Ca$^{2+}$ ion transport (6, 7). CD20 has no known natural ligand, and mice deficient in CD20 have no discernible perturbation of B cell development and function (8). Thus, CD20 is an appealing candidate for targeted therapies as it provides B cell specificity while sparing both hematopoietic stem cells and antibody-producing plasma cells.

Monoclonal antibody therapy: Rituximab

Rituximab is a chimeric mouse-human monoclonal antibody (mAb) consisting of a human kappa light-chain constant region, a human IgG1 Fc portion, and murine variable regions
which recognize human CD20 (huCD20). Upon binding to the CD20 antigen, rituximab’s in vivo anti-tumor effects are believed to result from several mechanisms, including induction of pro-apoptotic signaling, complement-dependent cytotoxicity (CDC), and antibody-dependent cellular cytotoxicity (ADCC) by Fc receptor-bearing effector cells (9-11) (Figure 1). An intriguing fourth possible mechanism involves the secondary recruitment of anti-tumor T cell effectors responding to tumor antigens released upon antibody-mediated tumor lysis (12-14). This cross presentation may lead to the expansion of cytotoxic T lymphocytes specific for lymphoma-associated antigens, producing a “vaccination” effect. The clinical important of this cross-presentation is suggested by data demonstrating that the induction of active immunity against lymphoma by vaccination with lymphoma-specific antigens can provide clinical benefits in some NHL patients (15, 16). All of these pathways are likely active in the clinical setting, but their relative contributions to the clinical effect of rituximab are still under investigation.

Figure 1. Proposed anti-tumor mechanisms of anti-CD20 monoclonal antibody. Rituximab binding to CD20 antigen in vivo is believed to induce apoptosis, complement-dependent cytotoxicity, antibody-dependent cellular cytotoxicity, and a secondary adaptive T cell response against tumor antigens released from dying lymphoma cells.
The first clinical phase I trial of single-agent rituximab was conducted in patients with relapsed indolent lymphomas and results established a tolerated dose of 375 mg/m^2 for a 4-dose weekly schedule (17). The subsequent phase II trial tested the efficacy of rituximab as a single agent and achieved an overall response rate of 48% with a complete response of 10% (18). These results led to the approval of rituximab by the US FDA in 1997 as the first monoclonal antibody therapy for the treatment of cancer. Since then rituximab has been tested in various NHL subtypes and achieved single agent response rates of up to 73% (19-21). Rituximab is routinely incorporated into all phases of conventional treatment, including first-line therapy, maintenance, and salvage therapy for various subtypes of NHL (22-24). Nonetheless, drug resistance to anti-CD20 antibodies eventually occurs in most patients, usually despite the continued expression of CD20 by tumor cells (9). Thus, strategies increasing the anti-tumor efficacy of anti-CD20 mAb therapies are required.

**Improving monoclonal antibody efficacy**

A number of promising approaches have been explored to enhance the effectiveness of anti-CD20 antibody therapy. Enhancing CD20 antigen expression may improve antibody-mediated killing, and it has been shown that histone deacetylase inhibitors can increase CD20 expression and thereby enhance the cytotoxic effects of rituximab (25). Furthermore, targeting anti-apoptotic members of the Bcl-2 protein family may favor apoptosis, and a phase II study combining antisense nucleotide oblimersen of Bcl-2 inhibitors with rituximab have reported a response rate of 60% in follicular NHL patients (26).

Most of the efforts have been focused at enhancing immune responses by augmenting CDC and ADCC. A study inhibiting CD59, the key membrane complement regulator that
inhibits CDC, reported enhanced anti-CD20 antibody-mediated CDC on resistant lymphoma cells \textit{in vitro} (27). ADCC seems to be the major mechanism of action \textit{in vivo}, and many studies have been aimed at activating Fc receptor-bearing natural killer cells, monocytes/macrophages, and granulocytes by systemic administration of cytokines such as α-interferon and IL-2 and growth factors G-CSF and GM-CSF (28-30).

Approaches targeting only one of the four mechanisms of action may be insufficient in increasing antibody efficacy as all four mechanisms are probably occurring simultaneously in the \textit{in vivo} setting of the host. Furthermore, systemic administration of immunomodulating agents may not achieve clinically relevant concentrations within the tumor bed. Thus, anti-CD20 antibody efficacy might be improved by linkage to small molecules which activate ADCC, antigen presenting cell functions, and T cell effectors within the tumor microenvironment. Delivering these immunostimulatory agents to tumor sites via validated antibody vehicles may not only promote great tumor cell killing, but also elicit secondary adaptive T cell immunity against tumor antigens released by dying tumor cells, leading to an “\textit{in situ} vaccination” effect.

\textit{Toll-like receptor (TLR) 9 agonist as immunostimulants}

Mammalian TLRs (TLRs 1-11) are expressed by host immune cells, where they serve to recognize pathogen-associated molecular motifs and stimulate innate and adaptive host antimicrobial immunity (31). TLR agonists are prime candidates for boosting anti-tumor immunity, with the TLR9 agonist CpG ODNs being the most extensively studied (32-35). There are at least 3 distinct classes of CpGs (A, B, and C), differing in their specific nucleotide sequences, backbone structure, and immunostimulatory activities. Class A CpGs induce the strongest secretion of α-interferon by plasmacytoid dendritic cells (pDCs), but little B cell
activation. In contrast, class B CpGs induce strong B cell proliferation and differentiation, but only modest $\alpha$-interferon secretion. Class C CpGs, possess unique structural features and combine immunomodulatory activities of both class A and class B CpGs. Since $\alpha$-interferon can be an active agent against B cell lymphomas (36, 37) and may improve clinical responses to rituximab (28, 38), achieving high-level induction of $\alpha$-interferon at tumor sites might be an important goal of antibody-CpG conjugates for improving immunotherapeutic efficacy.

**STUDY RATIONALE**

*Intratumoral delivery of CpG has potent activity against lymphomas*

Importantly, numerous studies suggest that local/intratumoral injection of single agent CpG may be superior to systemic administration in promoting anti-tumor immunity (39-43). Results from our laboratory in the highly-aggressive murine B cell lymphoma 38C13 stably transduced to express human CD20 (38C13-huCD20) have shown that direct intratumoral injection of CpG, but not systemic delivery, can significantly enhance the ability of rituximab to eradicate established tumors (44). Furthermore, in a phase I/II clinical trial of human class B CpG 7909, irradiation of a single peripheral lymphoma site followed by repeated weekly intratumoral injection of CpG led to regression of distant unirradiated tumor metastases and expansion of tumor-specific CD8+ T cells (45). Thus, intratumoral delivery of CpG to a site of killed human lymphoma led to an "in situ vaccination", with priming of a systemic T cell-mediated anti-lymphoma immune response. In sum, these data support the use of intratumoral delivery of CpG in combination with anti-CD20 antibodies.
Anti-CD20-CpG conjugates may target TLR9 agonist activity directly to tumor sites in vivo

The above results provide strong rationale for targeting CpG to tumor sites to improve rituximab efficacy. However, subjects with B cell lymphomas are unlikely to have all tumor sites accessible for repeated direct CpG injections. As an alternative to intratumoral injection, our laboratory has succeeded in linking CpG directly to anti-CD20 antibodies via a cleavable chemical linker, allowing antibody-mediated targeting of CpG to all tumor sites. Remarkably, preliminary data in the 38C13-huCD20 model have shown that anti-CD20-CpG conjugates eradicate 100% of 7-day established tumors in vivo (manuscript in preparation). This complete eradication was achieved with just two doses of anti-CD20-CpG conjugate, but not with equimolar doses of anti-CD20 antibody-control ODN, free CpG given i.v. or i.t., or control antibody-CpG.

Syngeneic, immunocompetent tumor models are required for preclinical evaluation of anti-CD20-CpG conjugates

The 38C13-huCD20 model may not adequately represent the physiology of human B cell lymphomas. First, 38C13-huCD20 cells are relatively insensitive to the anti-proliferative effects of CpG in vitro, whereas many human lymphomas exhibit sensitivity to this agent (manuscript in preparation). Second, the rapid tumor growth kinetics of 38C13-huCD20 may not allow enough time for the development of an adaptive immune response, limiting the ability to evaluate the effect of conjugate therapy on secondary T cell priming. Third, the model is not fully syngeneic as the expression of the target antigen, huCD20, is expressed only on the 38C13-huCD20 tumors and not on the host’s normal B cell compartment. Alternatively, the A20 lymphoma line, which arose spontaneously in the BALB/c strain, stably transfected with the huCD20 gene might serve
to recapitulate human B cell lymphomas, with sensitivity to direct CpG effects, slower growth rate, and capacity for induction of tumor specific T cell immunity (46). Furthermore, A20-huCD20 grown in huCD20-expressing BALB/c transgenic mice will serve as a fully syngeneic system to allow evaluation of therapy in the physiologic setting of huCD20 expression on normal B cells.

We hypothesize that anti-CD20 antibody efficacy may be improved by linkage to CpG to enhance ADCC, antigen presentation, and T cell responses within the tumor microenvironment. Thus the purpose of this dissertation project will be to characterize the activity and mechanisms of action for anti-CD20-CpG conjugates in the syngeneic immunocompetent A20-huCD20 model for B cell lymphomas through the following specific aims.

**SPECIFIC AIMS**

**Aim 1.** Generate and characterize anti-CD20-CpG conjugates with respect to stoichiometry and biological activity.

**Rationale and Methodology**

*Conjugation of CpG to anti-CD20 monoclonal antibody*

Anti-CD20 (or control IgG1) antibody was conjugated to 5’-amine-modified murine 1826 CpG (class B) or control 2138 CpG using the cleavable MHPH/4FB linker chemistry. **Figure 2** illustrates a schematic representation of the conjugation reaction.
Anti-CD20 antibody modification:

Rituximab at 10 mg/ml was exchanged into 1X MHPH modification buffer (0.1 M Phosphate Buffer, pH 7.2, 0.15 M NaCl, 0.05 M EDTA, 0.05% Azide) using a 7,000 MWCO Zeba Spin desalting columns. Rituximab was reduced under mild reducing conditions to selectively target hinge region disulfide bonds with 0.1 mM dithiothrietol for 1 hour at 37°C. The reduced antibody was then dialyzed back into 1X MHPH modification buffer. Ellman’s reagent was used to confirm successful antibody reduction. One mg of 3-N-Maleimido-6-hydrizinumypyrine hydrochloride (MHPH) was dissolved into 100 μl of DMF. Appropriate volume of linker was added to the reduced antibody according to the Solulink protein modification calculator, setting the equivalents of linker value to 7.5. This antibody-linker
mixture was incubated for 2 hours at room temperature. After buffer exchange into 1X conjugation buffer (0.1 M PBS, pH 7.2, 0.15 M NaCl, 0.05% sodium azide) using Zeba Spin desalt columns, the protein concentration was determined by BCA, and the antibody-MHPH linkage verified using the HyNic molar substitution ratio (MSR) determination protocol (Solulink).

1826 CpG modification:

Five mg of 5’-amino-labeled CpG 1826 ODN (sequence 5’-TCCATGACGTTTCCTGACGTT) was desalted into nuclease free water using a 5,000 MWCO VIVASPIN 500 spin filter unit, concentration determined by A\textsubscript{260} spectrophotometry, and CpG concentration yielding 0.5 OD \textsubscript{260}/μl in 300 μl final volume. C6-Succinimidyl 4-formylbenzoate (S-SS-4FB) was dissolved in dimethylformamide (DMF) at 100 mg/ml. The quantity of S-SS-4FB to be reacted with the CpG was determined using the amino oligo modification calculator supplied by Solulink, using the 1826 molar extinction coefficient of 181100. The entire volume of 5’-amino-labeled CpG was mixed with 20 equivalents of S-SS-4FB linker, plus one third volume of DMF, and incubated for 2 hours at room temperature. After centrifugation to remove any insoluble materials, the modified oligodeoxynucleotide in the supernatant was exchanged back into nuclease free water using VIVASPIN 500 spin filter units, and the final concentration adjusted to 0.3-0.5 OD 260/μl in 1X conjugation buffer. To quantify the 4FB modification level of the CpG, the molar substitution ratio of the modified CpG was determined according to the manufacturer’s instructions. Briefly, duplicate samples of 4FB-modified CpG were reacted with 2-hydrizinopyridine at 37\textdegree C for 30 minutes, and the A\textsubscript{350} of sample duplicates measured. Inputting the measured CpG concentration and the average of the duplicate A\textsubscript{350} values into the 4FB-MSR calculator was performed to confirm a molar
substitution ratio between 0.5 and 1.0.

Conjugation of anti-CD20-MHPH and CpG-4FB:

To complete the antibody-CpG conjugation, CpG-4FB and antibody-MHPH were mixed together and incubated for 14-16 hr at room temperature, in concentrations determined by the protein-oligonucleotide conjugation calculator (Solulink).

Purification of anti-CD20-CpG conjugates:

Conjugate was purified by buffer exchange into 1X PBS using a 30,000 MWCO VIVASPIN 15 column, then fast protein liquid chromatography (FPLC) using the ÄKTApurifier™ with GE Superdex 200 Size exclusion column and collection of fractions expressing an overlapping A\textsubscript{260}, A\textsubscript{280}, and A\textsubscript{354} signal. Concentrated fractions were pooled and the purified antibody-CpG conjugates quantitated using BCA to determine protein content and A\textsubscript{260} for DNA content. Stoichiometry calculations indicated that conjugates contained an average of approximately 4 CpG/ODN molecules per antibody molecule.

Characterization of anti-CD20-CpG conjugates

Stoichiometric analysis of the conjugates will be important for maintaining consistency between batch preparations and for dosing and scheduling determinations for \textit{in vivo} efficacy experiments. Relative stoichiometry of conjugation was initially determined by A\textsubscript{260} of CpG and protein concentration confirmed by BCA assay. The exact antibody to CpG ratio will be determined by employing isoelectric focusing and anion exchange chromatography methods. The anticipated ratio is one to three CpGs per one antibody, given the steric constraints of the free sulfhydryl groups in the hinge region and the electrostatic repulsion of the highly acidic CpGs. The biologic activity of conjugates was confirmed by a) binding to A20-huCD20 cells
Results and Discussion

**FPLC purification removes unconjugated CpG from anti-CD20-CpG conjugate**

Size exclusion chromatography (SEC) was used to purify the anti-CD20-CpG conjugate from unconjugated CpG (Figure 3).

![Figure 3](image)

**Figure 3. Size exclusion chromatography removes unconjugated CpG from anti-CD20-CpG conjugate preparations.** Concentrated rituximab or conjugate preparations were run through a Superdex 200 SEC column. X-axis represents elution time in minutes. A) FPLC profile of unconjugated rituximab, which elutes at 26 minutes. B) FPLC profile of conjugate reveals successful separation of unconjugated CpG from anti-CD20-CpG conjugate.

The FPLC profile for the unconjugated anti-CD20 antibody completely lacks the A$_{354}$ conjugate bond signal and elutes at 26 minutes with a high A$_{280}$ protein signal (Figure 3A). The FPLC profile of the anti-CD20-CpG conjugate reveals two distinct fractions, indicating successful separation of the conjugate from the free, unconjugated CpG (Figure 3B). The first fraction elutes at 23 minutes and contains the conjugate, as indicated by the strong A$_{354}$ conjugate bond signal. The second fraction elutes at 31 minutes and contains the unconjugated CpG, as it lacks the conjugate bond signal and has a high A$_{260}$ DNA signal. The high A$_{280}$ signal in the unconjugated fraction is from bleed-through artifacts between the A$_{260}$ and A$_{280}$ signals. Upon concentration and quantitation of FPLC purified conjugates, the final recovery of the
conjugated CpG has been in the range of 13-20%. Current efforts are being made to increase the efficiency and reproducibility of the conjugation reaction.

**Determination of CpG to anti-CD20 antibody stoichiometry**

FPLC purified anti-CD20-CpG conjugate and unconjugated anti-CD20 antibody were analyzed by SDS-PAGE, isoelectric focusing (IEF), and anion exchange chromatography (AEX) to visualize the different species of conjugates in the preparation. It would be of importance to determine how much unconjugated antibody there is in the conjugate preparation, and of the conjugated species, how many contain one, two, three, four (attached to the hinge region cysteine residues) or more. Given the mild reducing conditions of the antibody reduction and steric hindrance in the hinge regions, we speculate there to be three to four CpGs per antibody.

8% Tris-Glycine gels were run to allow maximum separation of proteins in the 250-140 kDa range to visualize differences between the conjugate preparation and unconjugated antibody as we suspected the conjugate to be larger in size due to the addition of CpG (Figure 4A). However, there were no significant differences in the upper range size range between the conjugate preparation and unconjugated antibody under non-reducing conditions. The lack of visible difference may be due to a low concentration of such species in the overall conjugate preparation or the size differences are too small to be detected.

The conjugate and unconjugated antibody were analyzed by IEF using pH 3-9 gradient gels to separate the conjugate species based on isoelectric points (pI) (Figure 4B). Lanes 1 and 3 containing the conjugate has several faint bands as indicated by the arrowhead, whereas Lanes 2 and 4 containing the unconjugated antibody has only one band at the upper limit of the gel. The band in the upper limit of the gel in all sample lanes appears to be the unconjugated antibody, as
the pI of rituximab has been reported to be pI 8.68 in literature (47). We suspect the multiple faint bands in Lane 1 and 2 to be the different species of conjugate as it is consistently present from samples containing only the conjugate preparation.

Figure 4. SDS-PAGE and isoelectric focusing analysis of anti-CD20-CpG conjugates. A) 8% Tris-Glycine SDS-PAGE gel of reduced and non-reduced conjugate and rituximab loaded with two different protein concentrations. No detectable difference in the upper range of the gel between the conjugate and rituximab. B) pH 3-9 gradient gel of conjugate (Lanes 1 and 3) and rituximab (Lanes 2 and 4). Faint bands in the acidic range of the gel are only apparent in lanes containing conjugate, indicated by arrowheads. Arrow illustrates the direction of sample migration.

Anion exchange chromatography resolves the different species within anti-CD20-CpG conjugate preparations

FPLC-purified rituximab and anti-CD20-CpG conjugates were analyzed by anion exchange chromatography using a MonoQ anion exchange column to detect the different species of conjugates (Figure 5).
Equilibration of the MonoQ column with pH 7.4 solution was able to elute the unconjugated antibody before the start of the gradient, as the pI of rituximab is greater than the pH (Figure 5A). The profile reveals small peaks starting at 8 column volume (CV), which we suspect to be antibody fragments. A 20 CV gradient with 2 M NaCl was able to separate the unconjugated antibody from the conjugate, which eluted off at 15 CV (Figure 5B). Furthermore, three distinct species of conjugates were resolved. Each contained the 354 nm conjugate bond signal and eluted at 15, 19, and 22 CV. Fractions containing the unconjugated antibody and distinct conjugate species will be further analyzed by gel electrophoresis and single stranded DNA staining to identify the species containing different antibody:CpG ratios.

**Biological activity of anti-CD20-CpG conjugate is maintained**

Antibody binding of the conjugate was compared to unconjugated anti-CD20 antibody by staining huCD20 positive 38C13 cells. The presence of CpG on the conjugate did not interfere with antibody binding, as the conjugate had comparable binding to rituximab (Figure 6A). CpG
is known to induce upregulation of costimulatory molecules, such as ICAM-1. Hence, the biological activity of the CpG on the conjugate was assessed by comparing upregulation of ICAM-1 on A20 cells pretreated with anti-CD20-CpG conjugate or unconjugated CpG. CpG on the conjugate maintained biological activity, as ICAM-1 was upregulated comparably to the unconjugated CpG (Figure 6B).

Figure 6. Biological activity of anti-CD20-CpG conjugate is maintained. A) Conjugate maintains antibody binding comparable to rituximab. A20-huCD20 cells were stained with 1, 0.1, or 0.01 μg of either rituximab or conjugate (normalized to antibody content) and detected with an anti-human kappa secondary antibody. B) Conjugate maintains ability to upregulate ICAM-1 expression of cells comparable to free CpG. A20 cells were incubated 48 hours with 10 μg/ml free CpG or conjugate (normalized to CpG content) and stained with anti-mouse ICAM-1 antibody.


Rationale and Methodology

Examination of cellular uptake and molecular trafficking of conjugates

Cellular uptake and molecular trafficking of conjugates will be important to examine, as it will allow us to better understand the molecular mechanisms of anti-tumor immunity. It will be of interest to determine if and when the CpG is liberated from the antibody once it reaches the tumor bed. If the CpG is liberated, is it subsequently up taken by the tumor cells themselves or by infiltrating immune cells? What is the identity of such cells that uptake the liberated CpG? Cellular uptake and molecular trafficking of conjugates will be assessed to answer such types of questions. 38C13 or 38C13-huCD20 cells were stained with anti-CD20-CpG-Alexa 488
conjugate (3 μg/ml CpG 1826 content) for 15 minutes at 4°C. Binding of conjugate to human CD20 was confirmed by flow cytometric analysis. Cells were cultured and stained for 24 hours at 37°C or 30 minutes at 4°C with 3 μg/ml CpG-FITC, or anti-CD20-1826-Alexa 488 conjugate (3 μg/ml CpG content). Cells were harvested and washed to remove unbound CpG or conjugate. To visualize the membrane, cells were stained with 4 μg of anti-CD19-APC for 30 minutes at 4°C, washed in 1X PBS, fixed with 2% paraformaldehyde, and adhered to glass coverslips. Coverslips were mounted onto glass slides with antifade reagent, and cured for 24 hours at room temperature before imaging. Images were captured with a confocal microscope using a 63X/1.4 oil objective. Leica Confocal Software was used to acquire images. If the conjugates are directly up taken by the tumors, intracellular trafficking will be examined by co-localization studies with the use of endosomal and lysosomal markers dextran and Lamp-1, respectively. In the absence of direct tumor uptake, it is anticipated that the CpG will be liberated from the cleavable linker and be up taken by effector cells present in the tumor microenvironment. The effect of CpG on these subsets of cells will be addressed in the mechanistic studies proposed in Aim 4. Furthermore, CpG conjugation to antibodies targeting internalizing antigens, such as CD19 which is also highly expressed on B cell lymphomas, may provide higher therapeutic efficiencies and will be compared against anti-CD20-CpG conjugates.

Results and Discussion

Fluorescently-labeled CpG conjugate is up taken by huCD20+ mouse lymphoma cells

Alexa 488-labeled CpG was used in the conjugation reaction to generate Alexa 488-labeled anti-CD20 CpG conjugates to examine cellular uptake and molecular trafficking. The Alexa 488-labeled conjugate maintains antibody binding and antigen specificity as confirmed by flow cytometry (Figure 7A). Initially, 38C13-huCD20 cells were incubated with free Alexa
488-labeled CpG for 24 hours to confirm cellular uptake detected by confocal microscopy. As shown in the top panel of Figure 7B, the Alexa 488 signal in the overlay image is located within the anti-CD19-APC stained membrane, confirming that the fluorescently-labeled CpGs are up taken by the cells. A short incubation of 38C13-huCD20 cells with the Alexa 488-labeled conjugate at 4°C (to inhibit cellular endocytosis) was able to bind cells, as indicated by the colocalization of the Alexa 488 and APC signals in the overlay images (Figure 7B, middle panel). Interestingly, after a 24 hour incubation, majority of the Alexa 488-labeled conjugates appeared to be up taken by the cells or bound to the membrane in distinct clusters (Figure 7B, bottom panel). Parallel analyses were conducted on huCD20− 38C13 cells and results revealed that conjugate binding and uptake was huCD20-specific.
Figure 7. **Cellular uptake of anti-CD20-1826-Alexa 488 conjugate.** A) Binding of conjugate to human CD20 was confirmed by flow cytometric analysis. 38C13 or 38C13-huCD20 cells were stained with anti-CD20-1826 CpG-Alexa 488 conjugate (3 μg/ml CpG content) for 15 minutes at 4°C. B) Binding and uptake of anti-CD20-CpG conjugate was assessed by confocal microscopy. Cells were cultured and stained at the indicated time and temperature with 3 μg/ml CpG 1826-FITC, or anti-CD20-1826-Alexa 488 conjugate (3 μg/ml CpG content). Images were captured with a confocal microscope using a 63X/1.4 oil objective. Leica Confocal Software was used to acquire images. Overlay images represent maximum intensity projections of 15 z-sections with 0.310 μm intervals. Scale bar: 25 μm.
These results imply that the CpG on the conjugate is able to enter cells, whereby it may directly exert its anti-tumor effects. To confirm that the Alexa 488-labeled conjugates are localized within the membrane, cells could be acid-washed after the 24 hour incubation to remove any bound conjugate and analyzed by confocal microscopy. Alexa 488 signal detected within the membrane would confirm CpG uptake. Rituximab is known to stabilize and redistribute CD20 molecules into lipid rafts on the surface of B cells, and the observed clustering of the Alexa 488 signal may represent lipid raft formation (48). Cells could be co-stained with lipid raft markers, such as ganglioside M1, and assessed by confocal microscopy whether the conjugate enhances lipid raft formation. Rituximab-CD20 complexes on lipid rafts have been shown to bind more strongly to C1q leading to higher levels of CDC and could be a possible mechanism of action of increased anti-tumor activity (49). Furthermore, rituximab has been shown to modulate CD20 from the cell surface through internalization, and it would be of interest to investigate whether the Alexa 488-labeled CpG that is up taken by the cell is still conjugated to the antibody or if it has been cleaved (50).

**Aim 3.** Evaluate the efficacy of anti-CD20-CpG conjugates in depleting the normal B cell compartment in huCD20 transgenic mice.

**Rationale and Methodology**

*Evaluate the ability of conjugates to deplete normal B cells*

As a prelude to treating huCD20-expressing mouse A20 lymphoma cells, we first sought to determine the effect of anti-CD20-CpG conjugates on the normal B cell compartment. B cell compartments were evaluated by measuring CD19$^+$ B cells in the blood, spleen, lymph nodes, and peritoneal cavity of huCD20 transgenic animals by flow cytometry (n = 3 mice/group). The
B cell-depleting activity of anti-CD20-CpG conjugates was compared to rituximab or PBS control one week post a single or double i.v. dose (one week apart) of equivalent antibody concentrations (151 μg). The B cell depletion studies would aid in determining the appropriate conjugate treatment dose and schedule in the transgenic animals. In some experiments, normal B cells may be pre-depleted with 150-250 μg of rituximab before therapy with conjugate, in order to remove the “sink” of CD20 target antigen on the normal B cell compartment that might otherwise lead to rapid clearance of the therapeutic anti-CD20-CpG conjugate (51).

**Results and Discussion**

*Conjugate has enhanced normal B cell depletion activity compared to rituximab*

A single dose of conjugate was able to achieve superior B cell depletion in the blood, spleen, and peritoneal cavity, while the level of B cell depletion in lymph nodes was comparable. After the second dose the depletion was superior in all compartments except peripheral blood (Figure 8).
Depletion with rituximab followed the hierarchy of B cell sensitivities to anti-CD20 antibody depletions reported in literature, whereby B cells in the blood were readily cleared and those in the peritoneal cavity were most resistant (52, 53). In contrast, depletion using the conjugate revealed a distinct pattern, with the greatest depletion efficiency in the peritoneal cavity. Double dose of rituximab resulted in a $1.8 \pm 15.7$ SEM fold decrease (high error can be attributed to sample processing) of peritoneal cavity B cells compared to the PBS control, whereas a double dose of conjugate achieved a nearly complete depletion.

A next step in these studies might be to investigate the mechanism of superiority of B cell depletion with conjugate treatment. It has been reported that macrophage immigration to the peritoneum may be the mechanism for B cell depletion in the peritoneal cavity (53). To address
this, animals could be treated i.p. with thioglycolate to induce macrophage migration into the peritoneal cavity, prior to depleting treatments, and observed for changes in B cell depleting efficiencies. It would also be of interest to investigate the durability of B cell depletion with conjugate treatment. This could be addressed by examining the B cell compartments longer than one week post the final treatment. Data from these experiments may provide valuable information regarding the mechanism of action for anti-CD20-CpG conjugates and enable appropriate treatment dosing and scheduling for in vivo experiments against experimental B cell lymphomas.

**SUMMARY & FUTURE DIRECTIONS**

Anti-CD20-CpG conjugates can be purified from unconjugated antibody and CpG while maintaining biological activity. Anion exchange chromatography resolved different species of conjugates, which may further be analyzed to determine the number of CpG molecules attached per antibody molecule. Changing this ratio may affect the anti-tumor efficacy of the conjugate, and should be optimized to maintain favorable pharmacokinetics while maximizing therapeutic potential. Confocal microscopy studies with fluorescently-labeled conjugates revealed cellular uptake on huCD20 expressing mouse lymphoma cells, which implied that the CpG is able to enter the cell even when targeting a non-internalizing target. Lastly, conjugates had greater normal B cell depletion efficiency compared to rituximab; even in more resistant B cell compartments such as the peritoneal cavity. These data provide strong rationale for not only evaluating conjugates for B cell lymphomas, but for disorders requiring depletion of B cells, such as autoimmune diseases.
Future studies should be directed at evaluating the *in vivo* efficacy of conjugates in syngeneic, immunocompetent huCD20 transgenic mice. Furthermore, conjugates containing CpG of the functionally distinct A, B, and C classes should be compared *in vivo*, as each class is known to have distinct immunomodulatory activities. Finally, the mechanisms of anti-tumor efficacy for the conjugates will need to be elucidated, with emphasis on direct tumor killing and secondary adaptive T cell responses. Data from these and future studies may yield exciting results, which may ultimately help warrant clinical evaluation of anti-CD20-CpG conjugate therapies in patients with B cell NHL.
REFERENCES


