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Research paper

Generation of tumor-targeted antibody–CpG conjugates

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ABSTRACT

A number of monoclonal antibodies against tumor-associated antigens have been developed for the treatment of cancer. The anti-tumor effects of such antibodies can be enhanced by conjugation to immune stimulatory ligands, such as the toll-like receptor 9 agonist CpG oligodeoxynucleotides (CpG). The present study describes methods for the conjugation of CpG to two clinically approved monoclonal antibodies (rituximab and trastuzumab) via a Sulfo-EMCS maleimide linker. This conjugation method yielded stable joining of CpG and antibody (molar range 2.2–4.3:1). Immunofluorescence studies showed intact antigen-specific antibody binding of the immunoconjugates, that were comparable to unmodified antibody. Furthermore, antibody–CpG conjugates demonstrated improved (rituximab) or equivalent (trastuzumab) immune stimulatory activity compared to free CpG *in vitro*. These studies demonstrate the feasibility of antibody–CpG immunoconjugates and provide the foundation for future *in vivo* immunotherapy evaluation.

1. Introduction

Conventional cancer treatment modalities, such as surgery, radiation therapy, and chemotherapy, remain limited in their ability to reach metastatic disease and eliminate cancer cells without collateral damage to normal tissues. Recently, monoclonal antibodies targeted to tumor-associated antigens or tumor growth factors have been developed for cancer therapy with the goal of overcoming these challenges. The inherent specificity of antibodies allows these reagents to be administered systemically while concentrating their cytotoxic or treatment effects in the tumor (Weiner et al., 2012). A number of tumor-targeted monoclonal antibodies have been used successfully for targeted cancer therapy, including trastuzumab for $Her2^+$, cetuximab and panitumumab for $EGFR^+$, rituximab for $CD20^+$, and alemtuzumab for $CD52^+$ malignancies (Deonarain, 2008). Since their advent in cancer therapy, several mechanisms of action have been characterized that contribute to their antitumor effects *in vivo*. By binding and blocking ligand-receptor growth and survival pathways, antibodies can elicit cell death directly (Weiner et al., 2012). In addition, engagement of the Fc portion of tumor cell-bound antibodies can trigger host innate immune effector mechanisms through Fc receptors (FcR), such as antibody-dependent cellular cytotoxicity (ADCC) and complement-mediated cytotoxicity (CMC) (Weiner et al., 2012). Modification of monoclonal antibodies to improve tumor cell killing also has been achieved through changes in the Fc portion to improve ADCC or CMC function and conjugation to radioisotopes or cytotoxic compounds (Steiner and Neri, 2011; Weiner et al., 2012).

Currently, ways to modify monoclonal antibodies to augment host anti-tumor immune responses have been explored for cancer immunotherapy. Immunotherapy uses the patient's immune system to recognize and eliminate tumor cells, with the potential to generate immunologic memory to prevent recurrent disease. In addition to improvement of ADCC and CMC activities, antibodies may be altered to stimulate or amplify cell-mediated anti-tumor immune responses. Blockade of regulatory checkpoints, like CTLA-4 or PD-1, can reverse

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tumor-driven immune tolerance and promote tumor killing (Simeone and Ascierto, 2012; Weiner et al., 2012). Furthermore, stimulation of immune effector cells can be achieved by cross-linking tumor antigens with T cells using bispecific antibodies (e.g. bispecific T cell engagers) or delivering additional immune stimulation through T cell co-stimulatory molecules, cytokines, and innate immune agonists (Lechner et al., 2011; Weiner et al., 2012).

In this report, we describe the use of the toll-like receptor (TLR) 9 agonist CpG oligodeoxynucleotides (CpG) conjugated to tumor-targeting antibodies as a new method to elicit an effective innate immune response at the tumor site. CpG contain motifs of unmethylated cytosine-phosphate-guanosine dinucleotides that mimic bacterial and viral DNA, and are known to stimulate dendritic cells, B cells, and natural killer cells (Ballas, 2007; Jakob et al., 1999; Krieg, 2002; Krug et al., 2001; Zhu et al., 2009). In addition, CpG therapy has been shown to elicit a Th1-like pattern of innate immune activation and inhibit immune suppressor cell populations like myeloid-derived suppressor cells (Shirota et al., 2012; Zoglmeier et al., 2011). These immune effects are mediated through TLR9-dependent and -independent pathways, and appear to be varied across species and the route of administration (Krieg, 2002; Krieg et al., 2004; Sanjuan et al., 2006). *In vivo* studies in experimental tumor models have shown that intratumoral delivery of CpG promotes survival and decreases tumor burden in murine models of melanoma (Amos et al., 2011; Buhtoiarov et al., 2011; Sharma et al., 2003), colon adenocarcinoma (Schettini et al., 2012; Sharma et al., 2003), and pancreatic adenocarcinoma (Schettini et al., 2012). Moreover, mice that achieved complete cancer remission with intratumoral CpG therapy were found to develop a protective memory response as indicated by resistance to tumor re-challenge (Sharma et al., 2003). However, the anti-tumor effects of free CpG require intratumoral delivery, as intraperitoneal and intravenous injections did not improve survival or significantly reduce tumor burden, likely due to its degradation and biodistribution away from the tumor site (Amos et al., 2011; Shirota et al., 2012). In addition, intratumoral CpG did not reduce tumor burden at remote uninjected sites (Amos et al., 2011), further illustrating the limitations of free CpG.

While intratumoral CpG has shown promise in preclinical investigations, translation of this approach to patients is limited by the fact that most cancers are not readily accessible. A more feasible approach to CpG immunotherapy is the development of tumor-targeted CpG reagents that may be given systemically. The present study demonstrates novel methods for the modification of clinically approved monoclonal antibodies with CpG with retention of both antibody binding and CpG bioactivity. By combining the tumor targeting features of monoclonal antibodies with the beneficial anti-tumor effects of CpG therapy, a potent new immunotherapy for cancer is possible.

2. Materials and methods

2.1. Cell lines and reagents

J7774A.1, Jurkat, MDA-MB-468, SK-BR-3, SK-OV-3, Raji, and Ramos cell lines were obtained from the American Type Culture Collection. D2F2/E2, a gift from Dr. Manuel Penechet (UCLA Medical Center, Los Angeles, CA), is a murine mammary tumor cell line stably transfected with human Her-2 (hu-Her-2). NS0-20 cells were generated by transfection of murine NS0 cells with human CD20 (hu-CD20). All cell lines were maintained in complete medium (RPMI-1640 supplemented with 10% fetal bovine serum, L-glutamine, penicillin G, and streptomycin) in humidified 5% CO_2, 37 °C incubators. Monoclonal antibodies trastuzumab (Herceptin) and rituximab (Rituxan) were purchased from Genentech (South San Francisco, CA). The hetero-bifunctional cross-linker N-[ε-maleimidocaproyloxy]sulfosuccinimide ester (Sulfo-EMCS) was purchased from Pierce Biotechnology. CpG oligonucleotide sequences were synthesized with a phosphorothioate linkage backbone structure for improved stability and nuclease resistance, and a modified C3 residue containing a thiol group to enable conjugation (Dalpke et al., 2002) by ValueGene (San Diego, CA). The previously published biologically active (human, murine) CpG-1826 sequence was used for these studies: 5′-TCCATGACGTTCCTGACGTT-3′ (Jakob et al., 1999).

2.2. Chemical conjugation

For chemical conjugation, purified antibody was combined with Sulfo-EMCS in 0.05 M phosphate buffer containing 1 mM EDTA (PBE; pH 7.4) and the mixture was incubated with continuous rotation at room temperature for 1 h. Conjugated antibody (antibody-EMCS) was concentrated and purified by Zeba™ Spin Desalting Columns [(7 K MWCO, 2 mL), Pierce, Rockford, IL] in PBE buffer.

Different ratios of antibody to EMCS were also evaluated over ranges of 1:5 to 1:10. Thiol-modified CpG were reduced with excess dithiothreitol (DTT, 0.1 M) in 1 mM EDTA, 20 mM Tris buffer (TE; pH 8.3–8.5) for 2 h at room temperature and protected from light. Subsequently, excess DTT was removed by Zeba™ Spin Desalting Columns. The activated CpG were joined to the linker-modified antibody (antibody-EMCS) by overnight incubation at 4 °C (molar ratio range of 1:5 to 1:10 evaluated), followed by quenching of residual reactive EMCS with L-cysteine. Unconjugated CpG were removed from the reaction by filtration chromatography on a Bio-Gel G60 gel column (Bio-Rad, Hercules, CA). The concentration of antibody–CpG conjugates was determined by UV spectrophotometry using the absorbance at 260 and 280 nm and the following equations:

$$\begin{aligned} A260 &= \varepsilon_{CpG260} \times C_{CpG} + \varepsilon_{mAb260} \times C_{mAb} \\ A280 &= \varepsilon_{CpG280} \times C_{CpG} + \varepsilon_{mAb280} \times C_{mAb} \end{aligned}$$

2.3. Gel electrophoresis and visualization

Purified antibody–CpG conjugates were confirmed by gel electrophoresis in 1.5% agarose by tandem visualization using ethidium bromide for nucleotide staining and Coomassie blue for antibody staining. The ratio of bound CpG to antibody was determined spectrophotometrically and calculated as an OD260/OD280 ratio, as developed previously by Judy Ngo and Shawna Oliva of TriLink Biotech (www.trilinkbiotech.com). Multiple batches of antibody–CpG conjugates were generated for this study with CpG to antibody ratios of 2.2-4.3.

2.4. Immunofluorescence

Binding of antibodies and antibody–CpG conjugates was evaluated by immunofluorescence techniques. NS0-20 or D2F2/E2 cells were seeded overnight in chamber slides. Prior to immunostaining, adherent cells were washed with PBS, fixed with acetone for 10 minutes at −20 °C, washed again with PBS, and then blocked for 1 h with 1% BSA in PBS. Cells were stained with antibody alone or antibody–CpG conjugates for 15 minutes at room temperature, using rituximab or conjugate on NS0-20 cells and trastuzumab or conjugate on D2F2/E2 cells. Specific binding was detected by incubation with a FITC-labeled secondary antibody (goat anti-human IgG-FITC, Life Technologies, Carlsbad, CA). Image acquisition was performed using an Axio Imager Zeiss Z1 fluorescence microscope (Axiovision Rel. 4.6.3, Carl Zeiss AG) connected to an automated, digital SPOT RTke camera and SPOT Advanced Software (SPOT Diagnostic Instrument Inc., http://www.diaginc.com). Images were resized for publication using Adobe Photoshop software (Adobe).

2.5. Flow cytometry studies

Antibody binding was also confirmed using flow cytometry. SK-BR-3, SK-OV-3, D2F2/E2, and MDA-MB-468 cells were used to test trastuzumab and trastuzumab–CpG conjugates. NS0-20, Raji, Ramos, and Jurkat cells were used to test rituximab and rituximab–CpG conjugates. Adherent cell lines were detached using Detachin (Genlantis, San Diego, CA) prior to staining. Possible FcR on NS0-20, Raji, Ramos, and Jurkat cell lines were blocked using purified mouse IgG2a, which can block both human and mouse FcR, for 10 minutes prior to antibody staining. Cells were stained with isotype control (human IgG1, clone A111), free CpG, antibody, or antibody–CpG conjugates in 2% FCS in PBS. Cells were washed twice with PBS and either stained with YOYO-1 (Life Technologies, Carlsbad, CA) for nucleotide staining or goat anti-human Fc-FITC (Life Technologies, Carlsbad, CA) for primary antibody detection. To detect CpG, YOYO-1 was added to samples on ice 5 minutes prior to running on a flow cytometer at a final working concentration of 0.25 μM. All samples were analyzed using the Attune Acoustic Focusing Cytometer (Life Technologies, Carlsbad, CA) and FlowJo software (Tree Star, Ashland, OR).

2.6. Immunoassay for bioactivity

Evaluation of CpG activity was performed by measuring cytokine production after *in vitro* treatment of murine monocyte/macrophage J7774A.1 cells. Briefly, J7774A.1 cells were cultured in complete medium in 96-well plates at 0.25×10^5cells/well and treated with antibody alone (rituximab or trastuzumab), antibody–CpG conjugate, free CpG, or vehicle alone. Concentrations of antibody–CpG conjugates were used to correspond to 1, 5, or 10 μg/mL CpG, and concentrations of antibody alone were equivalent to antibody concentrations of antibody–CpG conjugates. After 24 h, supernatants were collected and analyzed for IL-6 protein levels by ELISA (DuoSet mIL-6 ELISA, R&D Systems, Minneapolis, MN). Differences in mean IL-6 production among treatment groups were evaluated for statistical significance by ANOVA followed by Bonferroni-adjusted pair-wise comparisons. Data were analyzed and graphs were made using GraphPad Prism software.

3. Results

3.1. Generation of antibody–CpG conjugates

Since protein conjugation to the 3′end of CpG has been shown to maintain immunostimulatory activity of CpG, as compared to linkage through the 5′-end which negated potency (Kandimalla et al., 2002), CpG was linked to antibody at the 3′-end using a novel approach summarized in Fig. 1. Monoclonal antibodies, rituximab or trastuzumab, were conjugated to CpG using Sulfo-EMCS as a maleimide crosslinker. This approach enables conjugation with primary amine sites present on the surface of antibodies, minimizing undesirable polymerization or self-conjugation. In addition, the incorporation of a spacer arm in Sulfo-EMCS between the N-hydroxysuccinimide ester and the maleimide group limits steric hindrance. Subsequently, thiol-modified and activated CpG were conjugated to the antibody via the EMCS linkage. While numerous ratios of Sulfo-EMCS to antibody (from 5:1 to 10:1) and of CpG to the antibody-EMCS (from 5:1 to 10:1) were tested, the best yields of intact antibody–CpG conjugates were found using a 5:1 molar ratio of Sulfo-EMCS to antibody and a 5:1 molar ratio of CpG to antibody-EMCS. Gel column purification successfully isolated purified antibody–CpG conjugates from unconjugated reactants, as shown in Fig. 2.

To confirm that isolated conjugates retained both the CpG and antibody components, products were visualized by ethidium bromide and Coomassie blue stained in tandem for nucleotide and protein, respectively, under non-reducing conditions without SDS. As predicted, there is no ethidium bromide staining of the unconjugated antibody and no Coomassie staining of the free CpG, whereas antibody–CpG conjugate stained positively with both Coomssie blue and ethidium bromide (Fig. 3). In the Coomassie blue staining, the antibody–CpG conjugate appeared as a single band spanning the well. The broadness of this antibody–CpG band is likely due to slight variations in the number of CpG molecules conjugated per antibody. Inherent charge differences between the unmodified antibody, antibody–CpG conjugate, and free CpG in the absence of SDS are illustrated by the direction of migration in the gel. The unmodified antibody traveled towards the cathode, suggesting an overall positive charge, whereas the free CpG, which carries a negative charge due to the phoshorothioate backbone, traveled in the opposite direction towards the anode. The lack of migration of the antibody–CpG conjugate to either pole suggests a neutral charge.

3.2. Intact binding of antibody–CpG conjugates

Modification of monoclonal antibodies by chemical conjugation, like the addition of CpG, can alter their binding characteristics. The effect of CpG addition on antigen binding by rituximab and trastuzumab was evaluated using immunofluorescence and flow cytometry techniques. As shown in Fig. 4A, unmodified rituximab and rituximab–CpG immunoconjugates showed comparable specific staining of hu-CD20$^+$ NS0-20 cells.

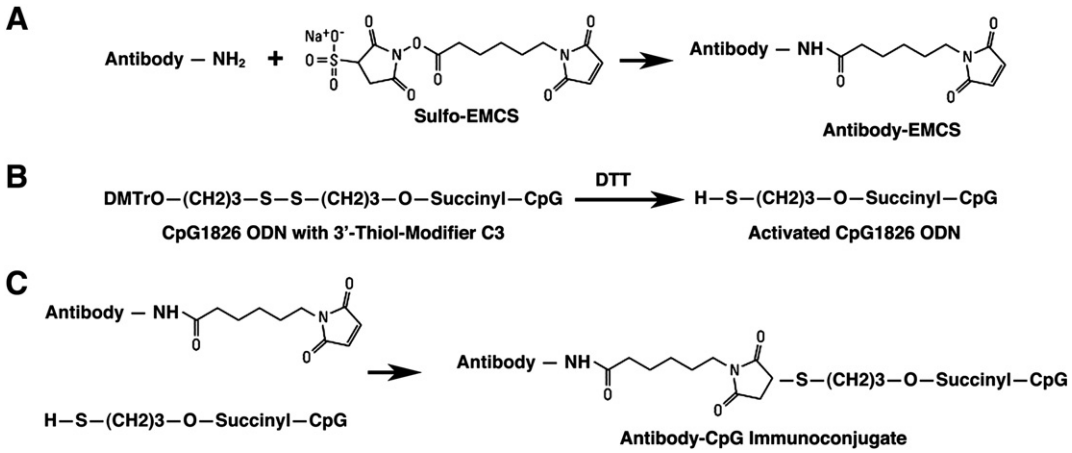

Fig. 1. Immunoconjugation reactions for the generation of antibody–CpG. Methods for the conjugation of CpG to monoclonal antibodies were developed and optimized. (A) Antibodies were first modified by the addition of an EMCS linker to allow CpG addition with minimal steric hindrance. (B) 3′ thiol-modified CpG ODN sequences were reduced prior to immunoconjugation with antibodies. (C) Activated CpG and antibody-EMCS were joined at a 5:1 molar ratio for optimal conjugation efficiency.

Similarly, trastuzumab–CpG immunoconjugates bound to hu-Her2$^+$ D2F2/E2 cells at levels comparable to native trastuzumab. While rituximab usually produces a ring pattern in CD20$^+$ lymphoma cells, transfection of CD20 into NS0 cells (NS0-20 cell line) was found to produce more of a speckled membrane immunofluorescence pattern possibly due to differences in expression of the CD20 antigen between transfected and endogenous expression. Non-specific binding was evaluated by immunofluorescence studies using trastuzumab–CpG with Her2$^-$ D2F2 cells and rituximab–CpG with CD20$^-$ NS0 cells. Neither antibody nor immunoconjugate showed positive binding to the negative cell lines (data not shown).

In addition to imaging studies, antibody–CpG conjugate binding was quantitatively analyzed by flow cytometry. Unlike imaging studies, flow cytometry allowed the detection of CpG bound to the cell surface, using nucleotide-staining dye, YOYO-1. Because YOYO-1 is membrane impermeable and has higher quantum yields when complexed to nucleotides, YOYO-1 can be used to detect nucleotides at the cell suface. CD20$^-$ human leukemia cell line Jurkat and CD20$^+$ human lymphoma cell lines Raji and Ramos were used to assess cell surface CpG in cells stained with rituximab–CpG conjugates (Fig. 4B). In CD20$^+$ cell lines, YOYO-1 staining is positive in cells stained with rituximab–CpG conjugates compared to cells stained with free CpG, isotype control, or unconjugated rituximab. By contrast, YOYO-1 staining in CD20$^-$ Jurkat cells was not different among isotype control, free CpG, rituximab, or rituximab–CpG stained cells. Correspondingly, Raji and

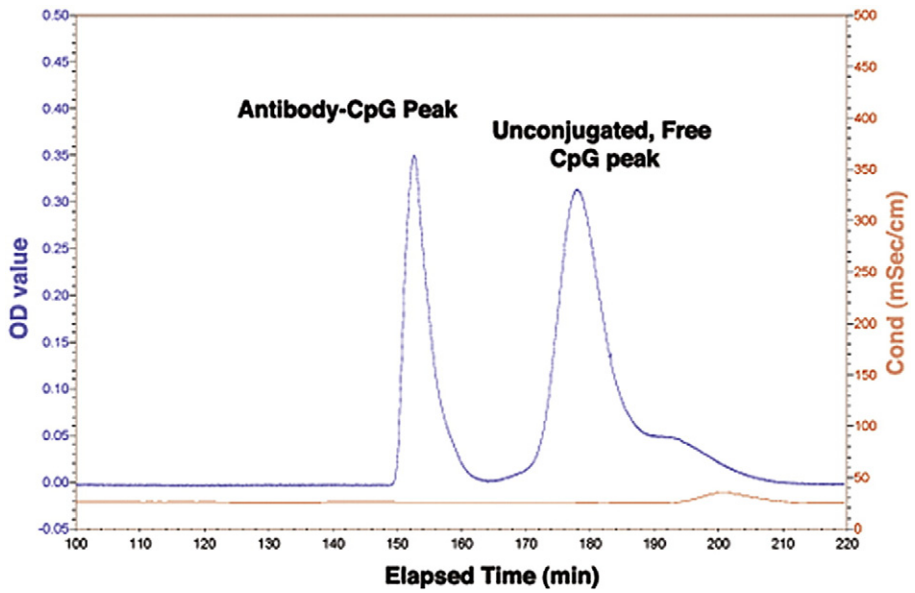

Fig. 2. Isolation of the antibody–CpG immunoconjugate from unconjugated, free CpG by column gel filtration.

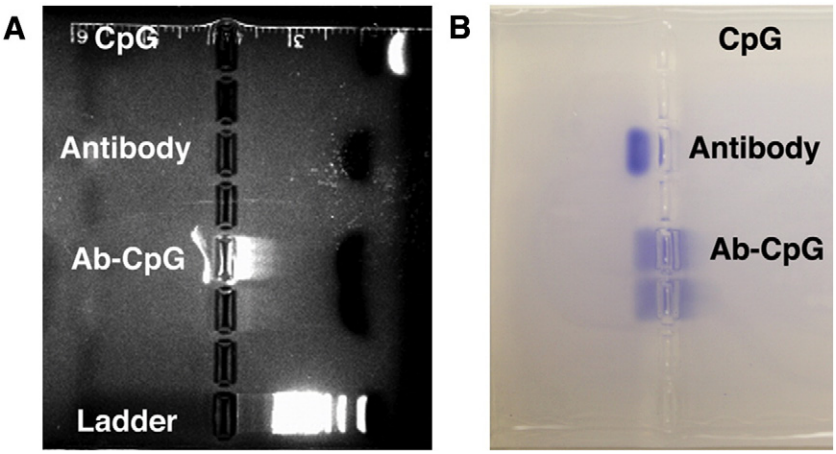

Fig. 3. Visualization of antibody–CpG immunoconjugates after agarose gel electrophoresis by tandem (A) ethidium brodmide and (B) Coomassie blue staining. Antibody–CpG immunoconjugates and unmodified components were electrophoresed on 1.5% agarose gels and subsequently stained for nucleotide and protein components.

Ramos are positive for both rituximab and rituximab–CpG binding, as detected by an anti-human Fc secondary reagent, while Jurkat cells were negative for staining. NS0-20 (hu-CD20$^+$), D2F2/E2 (hu-Her2$^+$), human breast cancer lines SK-BR-3 (hu-Her2$^+$) and MDA-MB-468 (hu-Her2$^-$), and human ovarian cancer line SK-OV-3 (hu-Her2$^+$) are adherent cell lines that could not be stained with YOYO-1 due to permeabilization of these cell lines after detachment from cell culture surfaces. However, positive FITC (antibody) staining was noted in rituximab and rituximab–CpG stained NS0-20, and in trastuzumab and trastuzumab–CpG stained SK-BR-3, SK-OV-3, and D2F2/E2 (data not shown).

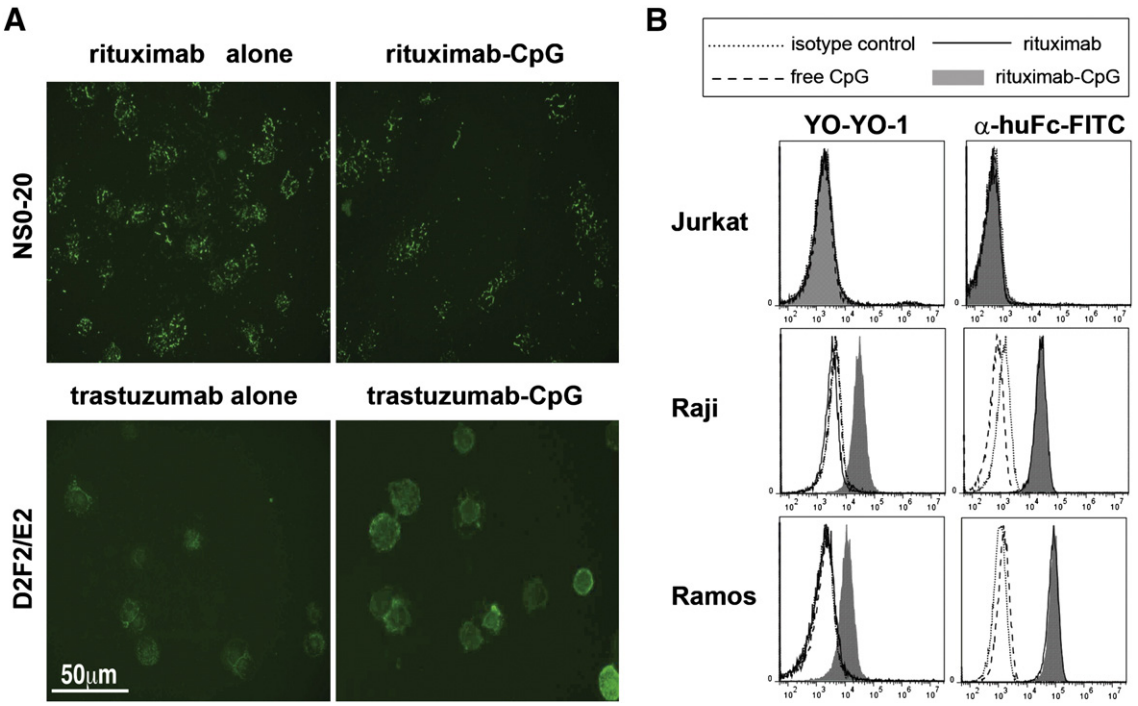

Fig. 4. Binding of native antibody and antibody–CpG immunoconjugates to antigen-positive cells by immunofluorescence techniques. (A) Specific binding of rituximab–CpG was comparable to unmodified rituximab antibody on hu-CD20$^+$ NS0-20 cells (original magnification 400×). Similarly, binding of trastuzumab–CpG immunoconjugates to hu-Her2$^+$ D2F2/E2 cells was comparable to binding by native trastuzumab antibody (original magnification 400×). (B) Flow cytometry histograms showing CpG at the cell surface (YOYO-1-positive cells) and antibody binding (FITC-positive cells). Only hu-CD20$^+$ cell lines stained with rituximab–CpG stained positively with YOYO-1 compared to isotype control, and only hu-CD20$^+$ cell lines stained with rituximab or rituximab–CpG were FITC positive.

3.3. Antibody-conjugated CPG ODN retain biologic activity

To evaluate whether conjugation to antibodies disrupted the immunologic activity of CpG, the ability of immunoconjugates to elicit IL-6 secretion by murine monocyte/macrophage J7774A.1 cells (hu-CD20$^-$, hu-Her2$^-$) was tested *in vitro* (Fig. 5). The biologic activity of CpG in the immunoconjugates was compared with free CpG and unmodified antibody, and treatment concentrations were normalized for CpG or protein content, respectively. Compared with untreated cells, free CpG and rituximab–CpG treatment produced a significant increase in IL-6 production at all doses ($p<0.01$). At doses of 5 and 10 μg/mL CpG, free CpG and rituximab–CpG caused significantly more IL-6 production than antibody alone ($p<0.01$). Interestingly, treatment with rituximab alone resulted in a small increase in IL-6 production compared to vehicle control ($p<0.01$). However, this effect was not dose-dependent, and is likely non-specific. It is possible that there may be some immunostimulatory effect due to Fc:FcR engagement, or other mechanisms that should be further investigated. While not statistically significant, at 5 and 10 μg/mL CpG, rituximab–CpG appeared more biologically active than free CpG, perhaps reflecting greater stability, cellular localization, and/or additive effects with FcR-engagement. Trastuzumab–CpG immunoconjugates similarly showed intact CpG activity, producing statistically significant increases in IL-6 production by J7774A.1 cells at 5 and 10 μg/mL of CpG ($p<0.01$). Unlike rituximab, trastuzumab alone did not significantly increase IL-6 compared to vehicle control, and trastuzumab–CpG showed equivalent activity to free CpG at all concentrations tested.

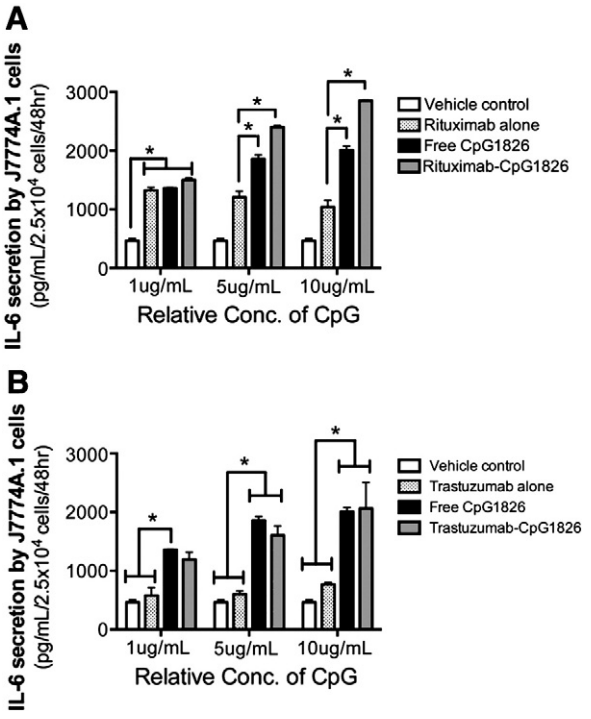

Fig. 5. Antibody-conjugated CpG1826 shows similar or increased biologic activity compared to free CpG1826. The biologic activity of CpG in immunoconjugates for (A) rituximab and (B) trastuzumab were evaluated by its ability to elicit IL-6 production by monocyte/macrophage J7774A.1 cells *in vitro*. Concentrations of CpG, antibody or immunoconjugate reflect the amount of CpG or equivalent. * indicates statistically significant differences in cytokine production between groups ($p<0.01$, $n=3$).

4. Discussion

Targeted cancer therapy using monoclonal antibodies has the potential to eliminate malignant cells, including occult and metastatic disease, selectively while minimizing effects on normal surrounding tissues. In addition to the inherent ADCC and CMC capabilities of some antibodies, modifications can improve the anti-tumor effects of these reagents. Conjugation of innate immune agonists, like TLR ligands, to tumor-directed antibodies can deliver potent immunotherapy stimuli to the tumor microenvironment to activate effector cells and reverse immune tolerance. While, intratumoral CpG delivery appears to be highly effective in eliciting anti-tumor immune responses *in vivo* (Amos et al., 2011; Buhtoiarov et al., 2011; Schettini et al., 2012; Sharma et al., 2003; Shirota et al., 2012), this approach is rarely feasible in cancer patients due to tumor location and/or widespread disease. However, delivery of CpG by antibodies that target tumor-associated antigens allows the reagent to be systemically administered (e.g. intravenously) and concentrated at tumor foci *in vivo*, the latter of which is required for CpG's innate immune effects.

The present study presents a method for the conjugation of tumor-targeted monoclonal antibodies to CpG that preserved both the specific binding and immune activity of both components. Schettini et al. (2012) reported the conjugation of CpG sequences to antibody for the immunotherapy of pancreatic cancer. The 4FB conjugation approach used by Schettini et al. (2012) yielded 5′ end joining of CpG to the antibody via a non-cleavable linker. While these conjugates produced tumor-killing *in vitro* and *in vivo*, these effects were attributed to TLR9-independent stimulation of NK cell ADCC because it was unlikely that antibody-5′-bound CpG had significant TLR9 activating effects. In contrast, the present study employed 3′ end joining of CpG to antibodies to preserve optimal CpG potency and TLR9 binding. Interestingly, rituximab also had some immnostimulatory effects and may have contributed to the immunostimulatory effects of the rituximab–CpG conjugate *in vitro*. On the other hand, trastuzumab alone did not have an immunostimulatory effect. Unlike humanized antibody trastuzumab, rituximab is a chimeric antibody, and it is possible that its murine regions may have some stimulatory effect on the murine macrophage cell line. Another possible mechanism for stimulation may include Fc:FcR interactions, which have been shown to be crucial for rituximab's anti-tumor effects in patients and mouse tumor models (Lim et al., 2010; Clynes et al., 2000). Further investigation is needed to explain why rituximab stimulated IL-6 secretion in the J7774A.1 cell line. However, it should be emphasized that rituximab's effect was relatively low and not dose-dependent, unlike effects seen with rituximab–CpG and CpG alone.

Future immunotherapy studies in experimental tumor models will evaluate the efficacy of these antibody–CpG conjugates *in vivo*. In addition to potentially concentrating CpG at the tumor site, conjugation of CpG to tumor-targeted antibodies may have additional beneficial effects. When the targeting antibody has significant cytotoxic activity (e.g.

ADCC), the resulting tumor cell death and release of antigens could act synergistically with CpG to promote anti-tumor immune responses. Furthermore, antibody targeting and binding to the surface of tumor cells compared to free CpG may enhance internalization of the CpG moiety to provide increased bioactivity due to activation of cytoplasmic TLR9. These studies demonstrate the feasibility of antibody–CpG immunoconjugates and provide the foundation for future *in vivo* immunotherapy evaluation.

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