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Targeted Activation of Toll-Like Receptors: Conjugation of a Toll-Like Receptor 7 Agonist to a Monoclonal Antibody Maintains Antigen Binding and Specificity

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Abstract:
Therapeutic activation of Toll-like receptors (TLR) has potential for cancer immunotherapy, for augmenting the activity of anti-tumor monoclonal antibodies (mAbs), and for improved vaccine adjuvants. A previous attempt to specifically target TLR agonists to dendritic cells (DC) using mAbs failed because conjugation led to non-specific binding and mAbs lost specificity. We demonstrate here for the first time the successful conjugation of a small molecule TLR7 agonist to an anti-tumour mAb (the anti-hCD 20 rituximab) without compromising antigen specificity. The TLR7 agonist UC-1V150 was conjugated to rituximab using two conjugation methods and yield, molecular substitution ratio, retention of TLR7 activity and specificity of antigen binding were compared. Both conjugation methods produced rituximab-UC-1V150 conjugates with UC-1V150 : rituximab ratio ranging from 1:1 to 3:1 with drug loading quantified by UV spectroscopy and drug substitution ratio verified by MALDI TOF mass spectroscopy. The yield of purified conjugates varied with conjugation method, and dropped as low as 31% using a method previously described for conjugating UC-1V150 to proteins, where a bifunctional crosslinker was firstly reacted with rituximab, and secondly to the TLR7 agonist. We therefore developed a direct conjugation method by producing an amine-reactive UV active version of UC-1V150, termed NHS:UC-1V150. Direct conjugation with NHS:UC-1V150 was quick and simple and gave improved conjugate yields of 65-78%. Rituximab-UC-1V150 conjugates had the expected pro-inflammatory activity *in vitro* (EC_{50} 28-53 nM) with a significantly increased activity over unconjugated UC-1V150 (EC_{50} 547 nM). Antigen binding and specificity of the rituxuimab-UC-1V150 conjugates was retained, and after incubation with human peripheral blood leukocytes, all conjugates bound strongly only to CD20-expressing B cells whilst no non-specific binding to CD20-negative cells was observed. Selective targeting of Toll-like receptor activation directly within tumors or to DC is now feasible.

Keywords: Toll-like receptors, TLR7, tumor immunotherapy, monoclonal antibody

Introduction

Therapeutic triggering of TLRs for vaccine and tumor immunotherapy

Pattern Recognition Receptors (PRRs), such as Toll-like receptors (TLRs), C-type lectins and Nod-like Receptors (NLRs) are germ line encoded transmembrane proteins pivotal in priming the activation

of the adaptive immune system.[1–4] A range of synthetic and biosynthetic agonists targeting TLR activation have been developed for use both as vaccine adjuvants and for cancer immunotherapy. [5–8] TLR agonists range in size from small molecules (imiquimod, guanisine and adenosine analogs) to large and complex biomacromolecules such as lipopolysaccharide (LPS), nucleic acids (CpG DNA, polyI:C) and lipopeptides (Pam_3CSK_4).

Currently of the 5 licensed human vaccine adjuvants, only adjuvant system 04 or AS04, contains a TLR agonist. This adjuvant is currently utilized in the human papilloma virus vaccine Cerivax® and hepatitis B vaccine FENDrix®.[9,10] AS04 contains a biosynthetic TLR4 agonist, Monophosphoryl lipid A (MPL), added to the conventional adjuvant alum.[11] In contrast to alum alone, AS04 promotes both Th1 and Th2 based responses, rather than a focused Th2 response.[12] Particulate formulations, such as alum, drive immunization by providing a localized deposition of antigen (Ag) but also through inflammasome activation via NLRP3 and producing IL-1β and IL-8 promoting Th2 responses.[13–15] Inclusion of the TLR agonist MPL activates additional cytokine responses including IL-12 production, that together lead to Th1 activation.[16]

Aldara is a cream containing the synthetic TLR7 agonist imiquimod, the only other synthetic TLR agonist currently licensed for human treatment. Its current applications are topical treatment of actinic keratosis, external genital warts and basal cell carcinomas,[17] demonstrating the broad potential for TLR activation for immunotherapeutic treatment of tumors and viral infections. As well as topical immunostimulation, imiquimod is also a candidate adjuvant, and has been shown to promote adaptive immune responses when applied topically to the site of immunization in studies involving a subunits from the parasite *Plasmodium falciparum*,[18] as well as improving the efficacy of subunit cancer vaccines.[19–21]

However, immunotherapy with imiquimod is currently limited to topical formulation, as localization of potently proinflammatory TLR agonists is vital. Indeed, a major concern with therapeutic use of any TLR agonist, which has to date highly limited their clinical use, is that systemic TLR activation can be fatal, with toxic shock caused by cytokine syndrome or cytokine storms.[22,23] Recent efforts have therefore focused on reducing and eliminating this systemic toxicity. Typical pro-drug and ante-drug formulations have had limited success in imparting tolerability to TLR 7 agonists.[24–26] Antedrugs are active compounds that are metabolically inactivated before entering systemic circulation [27]. An alternative approach is to limit drug availability and localize inflammation by covalent conjugation to macromolecular scaffolds such as proteins and polymers, which can effectively limit systemic cytokine levels but retain high levels of inflammation at the site of administration.[28] A further benefit of TLR agonist conjugation to proteins is the increase in adjuvant activity when TLR agonists are directly and covalently coupled to protein antigen over mixtures of antigen and agonists.[29] The success of protein conjugation of many TLR agonists raises the possibility of using mAbs to deliver TLR agonists.

Rationale for conjugating TLR agonists to monoclonal antibodies.

We identified three distinct therapeutic applications for TLR agonists conjugated to mAbs: to target therapeutic TLR activation to tumors; to promote mAb cytotoxicity; and to deliver TLR agonists to antigen presenting cells (APC).

As various immunomodulatory features of the tumor microenviroment have been identified,[30] it has become increasingly clear that selective triggering of TLRs at the site of a tumor can have both direct

and indirect therapeutic benefits. Driven by proven clinical efficacy against basal cell carcinoma,[31] the cancer therapeutic effects of TLR7 agonists such as imiquimod have been extensively characterized, and include induction of tumor cell apoptosis.[32–34] Likewise, the established therapeutic efficacy of intravesicular BCG for bladder cancer has led to investigations into replacing this complex biologic with one or more synthetic TLR agonist.[7,35] However, immunotherapy by deliberate TLR activation can only safely be achieved by localizing activation and subsequent proinflammatory signals, and so tumor targeting is essential. Conjugation to tumor localizing mAbs is a well-established method for delivering drugs to solid tumors.[36,37]

Even without conjugation to cytotoxic drugs, monoclonal antibodies (mAb) are well established cancer treatments,[38] with examples including cytotoxic mAbs targeting CD20 for treatment of lymphoma (Rituximab, MabThera), and antibodies targeting Her2 for treatment of Her2+ breast cancer (Trastuzumab, Herceptin). However, the efficacy of many mAbs is often limited by poor cytotoxicity, and direct tumor cell killing *in vivo* is also limited by the anti-inflammatory tumour microenviroment.[39] Improved tumor killing can be achieved using antibody-drug conjugates (ADC) that deliver cytotoxic drugs to tumors cells.[37] An alternative approach is to improve the cytotoxicity of mAbs. Through Fc region binding to FcγR on macrophages, antibody dependent cellular cytotoxicity (ADCC) can directly kill antibody-labelled tumor cells, but in the tumor microenvironment inhibitory FcγR dominate preventing ADCC. TLR activation at the tumor site is expected to overcome inhibitory FcγR and promote ADCC,[40] and therefore we propose that TLR agonists conjugated to anti-tumor mAbs would be expected to have enhanced tumor cytotoxicity. Similarly, TLR triggering is known to augment the effects of immunostimulatory mAbs such as anti-CD40, to synergistically promote CD8+ T-cell expansion independent of CD4+ T cells, by bypassing the requirement for helper T-cell triggering of DC through receptors such as CD40.[41] Again, the enhancement of antibody activity with TLR stimulation suggests that conjugation of TLR agonists to mAbs could be beneficial.

The only previous report of antibody-TLR agonist conjugates attempted to target a TLR9 agonist (CpG oligonucleotide) together with antigen directly to subsets of APC in order to develop improved anti-tumor vaccines and promote CTL priming. Antibody mediated targeting of antigen to subsets of APC can promote, modulate or inhibit adaptive immune responses,[42] especially since targeting antigen to DC does not inherently lead to the activation required to promote priming of adaptive immunity. Unfortunately, the biomacromolecular TLR agonists conjugated to DC-targeting mAbs in this study had physicochemical properties that were incompatible with specific antibody targeting, and thus antibody specificity was lost even though the conjugates retained proinflammatory activity, and still promoted CTL priming and induction of anti-tumor immunity.[43] TLR9 agonists such as CpG oligos are known to be bound by scavenger receptors and promote antigen presentation even in the absence of the TLR9 receptor[44] possibly related to its polyanionic structure.[45]

This report suggested that alternative TLR conjugation strategies are required to achieve specific targeting using antibody conjugation. Other nucleic acid TLR agonists, such as the TLR3 agonist polyI:C are highly polyanionic, and synthetic TLR2/4 agonists such as Pam3CSK4 contain a highly hydrophobic lipid tail. These classes of agonist are likely to bind to a range of cells and proteins and thereby compromise antigen specificity and targeting. In contrast small molecule TLR agonists such as the purine analogs or imidazoquinolines that activate TLR7 and/or 8 are far better suited to ADC

development as these small molecules lack any of the physicochemical features likely to compromise antibody specificity.

This study therefore aimed to investigate the feasibility of targeting small molecule TLR agonists using mAbs. To allow rapid evaluation of TLR7 conjugates of a range of different mAbs, we needed to develop a rapid, easy to quantify conjugation method that retains the native ability to bind antigens, but avoids creating non-specific binding, as well as retaining the characteristic pro-inflammatory TLR activation response seen with small molecule TLR7 agonists alone. We therefore synthesised the TLR agonist UC-1V150 **2** as it has well described protein conjugation methods.[28] Rituximab was chosen as a model anti-cancer mAb because it is well characterized, and allows rapid and simple evaluation of specific binding activity using human peripheral blood leukocytes (PBL) which contain both CD20-expressing cells together with a wide range of different CD20-negative cells. A simplified protein conjugation method was developed to improve yield of TLR7 agonist conjugates. We found for the first time that it is possible to make TLR7 agonist-mAb conjugates that both retained proinflammatory activity and specific antigen binding.

Results

Synthesis of amine-reactive TLR7 agonist NHS:UC-1V150

A published method[28] for conjugating UC-1V150 to proteins by labelling antibody with an amine-reactive bifunctional linker, followed by addition of TLR7 agonist UC-1V150, was initially tested with BSA and mouse and human mAbs. Although this 'indirect conjugation' method (Figure 1A) produced conjugates with BSA as expected, in contrast this method gave poor conjugate yields with mAbs and a significant degree of precipitation was observed suggesting protein denaturation or crosslinking (data not shown). This precipitation was highly dependent on the concentration of bifunctional crosslinker used (data not shown). An alternative method for conjugating TLR7 agonists to proteins was therefore proposed, referred to as 'direct conjugation' (Figure 1A) whereby the bifunctional linker (**1**) was reacted firstly with TLR7 agonist UV-1V150 (**2**) to produce the novel amine reactive TLR7 agonist termed NHS:UC-1V150 (**3**) (Figure 1B), which could then be directly conjugated to free amines on the antibody. UC-1V150 was reacted with *N*-(2,5-dioxopyrrolidin-1-yl)-6-(2-(propan-2-ylidene)hydrazinyl)nicotinamide using aniline as a catalyst yielding a 96% conversion by ^{1}H NMR. The resulting product contained UV characteristics of both UC-1V150 λ_{max} 278 nm and *N*-(2,5-dioxopyrrolidin-1-yl)-6-(2-(propan-2-ylidene)hydrazinyl)nicotinamide λ_{max} 340 nm shifted from 320 nm. Monitoring and characterisation of the reaction by NMR indicated that the aldehyde proton δ 9.98 decreased proportionately with the dimethyl protons of *N*-(2,5-dioxopyrrolidin-1-yl)-6-(2-(propan-2-ylidene)hydrazinyl)nicotinamide at δ 2.00. The hydrolysis reaction, yielding the free hydrazine and acetone as a byproduct, occurred as a result of the condensation aniline with the aldehyde, indicated by the brief formation of the enanime intermediate seen at δ 8.59. The appearance of singlets at δ 11.83 and 8.16 indicated the formation of the hydrazone. Once the reaction had gone to completion, the mixture was stored in a dessicator to avoid hydrolysis, and used without further purification to conjugate directly to mAbs (direct method; Figure 1A).

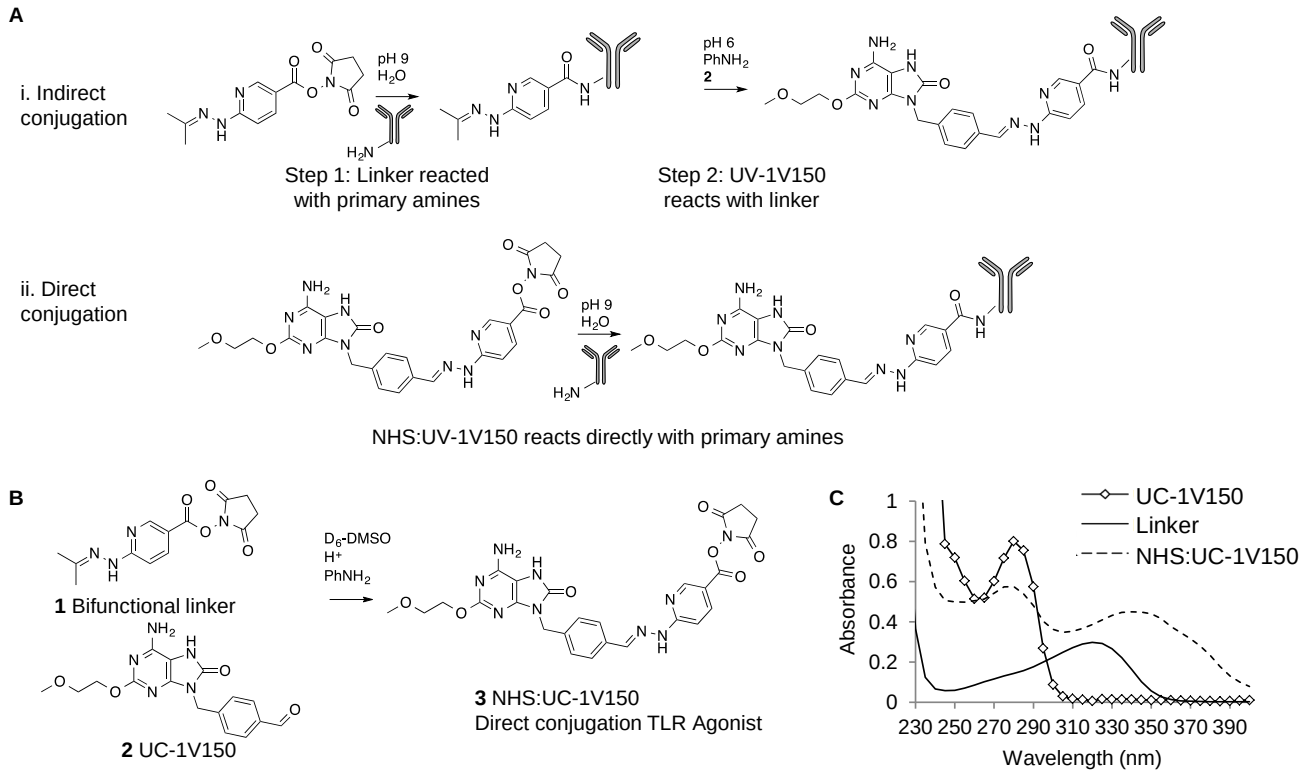

Figure 1 Synthesis of NHS:UC-1V150 for direct conjugation to proteins compared to conventional indirect conjugation protocol.

A Reaction schemes for conventional indirect conjugation method (i) vs new direct conjugation protocol (ii). **B** Synthetic scheme used to produce amine reactive TLR7 agonist **3**, from bis-functional crosslinker **1** and TLR7 agonist UC-1V150 **2**. **C** UV spectra of linker **1**, TLR7 agonist UC-1V150 **2** and resulting amine reactive TLR7 agonist for direct conjugation NHS:UC-1V150 **3**.

Conjugation of rituximab to TLR7 agonist UC-1V150

Protein-UC-1V150 conjugates have been described, but conjugation of TLR7 agonists to an antibody has not previously been demonstrated. We compared two alternative conjugation methods (Figure 1A), and found that both were able to produce TLR 7 antibody conjugates with varying substitution ratios (Table 1; Figure 2). Yields varied significantly with the direct conjugation provided improved yields. Yields in both methods were substitution ratio dependent suggesting conjugation caused the antibodies to become unstable (Figure 2C). In particular protein yield in the indirect conjugation method suffered significantly from increasing substitution ratio above 1:1. As intended, the new direct conjugation method gave an improved yield of conjugate compared to the indirect method (Figure 2C), and precipitation was much reduced (data not shown).

Table 1: Conjugation conditions used to produce TLR7 agonist – rituximab conjugates studied

	Molar Equivalents			
Sample	Rituximab	Bifunctional crosslinker **1**	UC-1V150 **2**	NHS:UC-1V150 **3**
Indirect (Low MSR)	1	2	8	0
Indirect (High MSR)	1	5	8	0
Direct (Low MSR)	1	0	0	10
Direct (High MSR)	1	0	0	20

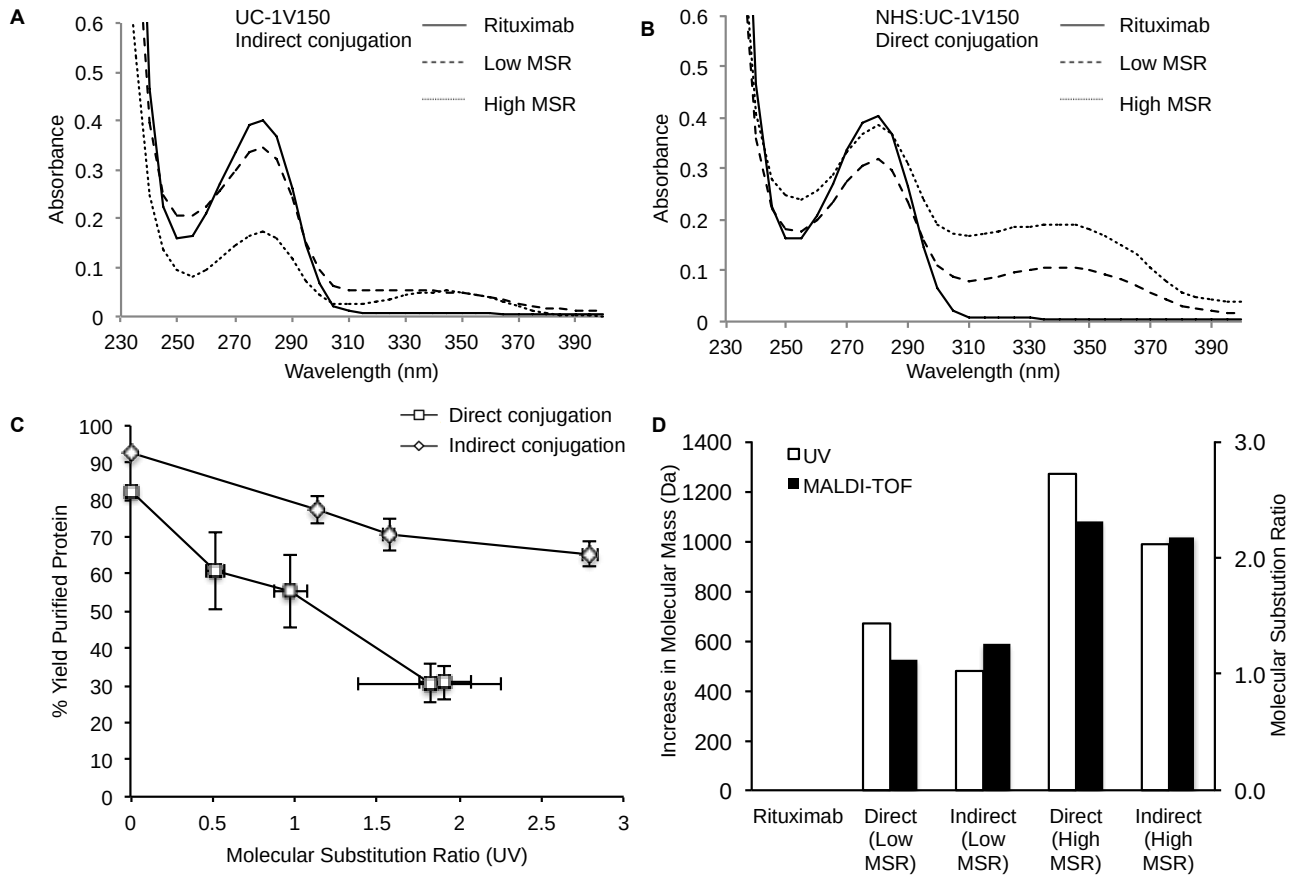

Figure 2 Characterisation of rituximab-TLR7 agonist conjugates for drug loading and yield of purified conjugate.

A,B UV spectra of rituximab-UC1V150 conjugates produced using indirect (**A**) and direct protocols (**B**). **C** Comparison of protein yield after purification for conjugates produced using indirect vs direct protocols. **D** Comparison of mass increase of Rituximab UC-1V150 conjugates observed by MALDI-TOF/TOF mass spectroscopy vs conjugation ratio calculated from UV spectroscopy (A_{350}).

Quantification of TLR7 agonist loading

After conjugation of the TLR 7 agonists and subsequent separation of conjugated from unconjugated drug by size exclusion chromatography, the amount of UC-1V150 covalently coupled to rituximab was analysed to determine firstly if conjugation had occurred and secondly to monitor the efficiency and

effect of conjugation. UV absorbance (A_{340}) was initially used to determine concentration of the bis-aryl hydrazone system formed in the conjugation process shown by the altered UV profile of conjugates (Figure 2 A and B). The discrepancy in λ_{max} A_{340} and A_{280} from the altered UV profiles of conjugates produced by direct and indirect methods reflects the variation in yield observed between the two conjugation methods (Figure 2 C). Once concentration of the hapten system was known, a mass increase over the native protein was calculated and compared to data from MALDI-TOF/TOF mass spectroscopy analysis of the samples (Figure 2 D). Both UV and MALDI-TOF/TOF concluded a similar mass increase over the native protein indicating conjugation had occurred, substitution ratio was scalable and confirming the accuracy of UV absorbance for rapid conjugate analysis.

Pro-inflammatory activity of rituximab TLR 7 agonist conjugates

Purine analogues have been long known for potent proinflammatory activity, which can be attributed to triggering TLR7 and/or TLR8.[46] Conjugation of these and similar compounds to macromolecules including proteins and polymers increase the relative potency of the active compound. We monitored IL-12p40 secretion from RAW 264.7 to measure proinflammatory activity of compounds, and determine if conjugation of UC-1V150 to antibodies affected proinflammatory responses, and if the expected increase in potency was observed. UC-1V150 produced a pro-inflammatory response with an EC_{50} of 547 nM based on IL-12p40 concentration (Table 2). Rituximab and mock conjugates without UC-1V150 failed to produce a response in this assay at the highest concentration tested (Figure 3), confirming the mAb and conjugation procedure were free from proinflammatory contaminants. Simply mixing UC1V150 with rituximab did not alter UC-1V150 activity (data not shown). Both direct and indirect conjugation methods produced conjugates that powerfully induced IL-12p40 production, and showed greatly increased potency over the unconjugated UC-1V150, with potency correlating closely to UC-1V150 concentration (Figure 3A and B). The low conjugate yields obtained using the indirect conjugation method at higher crosslinker concentrations provided insufficient quantity of this conjugate to fully characterize activity, and therefore it was not possible to evaluate the potency *in vitro* of high substitution ratio indirect conjugate.

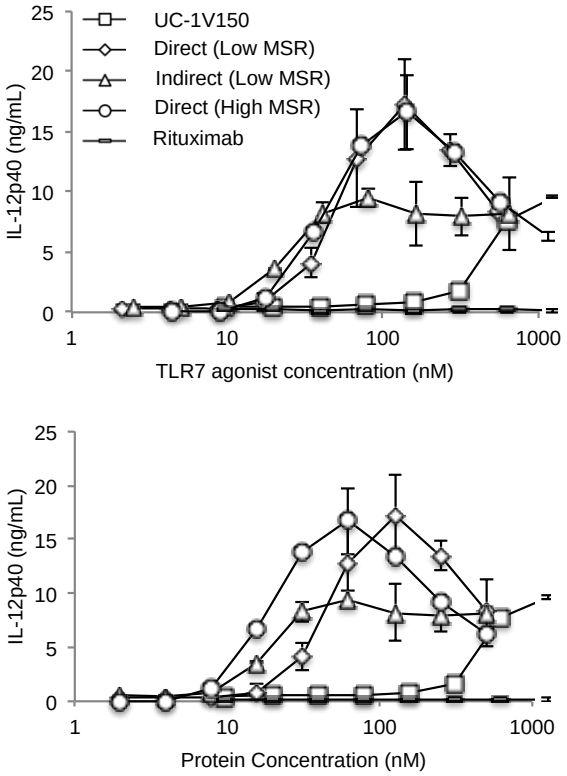

Figure 3 *In vitro* analysis of TLR7 agonist activity of Rituximab UC1V150 conjugates.

IL-12p40 secretion by RAW 264.7 stimulated with conjugates was plotted against drug concentration (top) or protein concentration (bottom).

Table 2: Influence of Molecular Substitution Ratio of TLR7 agonist - rituximab conjugates on proinflammatory activity.

Sample	Molecular Substitution Ratio (TLR7 agonist : rituximab)		IL-12 stimulation EC_{50} (nM)
	UV absorbance	MALDI-TOF mass spectroscopy	
Rituximab	0	0	>2000 ± 0
Indirect (Low MSR)	1	1.3	28 ± 3
Indirect (High MSR)	2.1	2.2	Not Tested
Direct (Low MSR)	1.5	1.1	53 ± 8
Direct (High MSR)	2.8	2.3	31 ± 3
UC-1V150	NA	NA	547 ± 47

Specific binding to CD20 expressing cells in human peripheral blood leukocytes

In a previous study significant loss of antibody specificity was observed when TLR agonists were conjugated to antibodies that targeted DC.[43] During the conjugation process, some loss of protein was observed with some precipitation visible, especially in conditions where higher concentrations of cross-linker were used and when the indirect conjugation method was used. This raised concerns over possible effects of conjugation on the mAb affecting structure or adding steric bulk. For antibodies this could modify or reduce antigen binding or increase non-specific binding.[43] In order to measure binding of TLR-mAb conjugates, a Zenon staining protocol was utilized. This relies on complexing the rituximab, rituximab conjugates, or an isotype control, to purified fluorescently labelled Fab fragments of secondary antibodies against human IgG prior to addition to PBL. This has the advantage of avoiding any requirement to covalently label antibodies and conjugates with fluorophores either before or after conjugation, which could affect binding or be affected by conjugation, and simply staining with secondary antibodies was also not possible for staining human PBL such as B cells that are already coated with human IgG. Leukocytes from human peripheral blood were first stained with APC anti CD19 and FITC anti CD3 to identify CD20-positive B cells and CD20-negative T cells respectively (Figure 4 A).

After fluorescent complexes of rituximab, rituximab-UC-1V150 conjugates and an isotype matched control antibody were formed using the PE Zenon human IgG1 reagent, these were incubated with the CD19/CD3 double stained PBL, which were then washed, fixed, and analysed by flow cytometry. Unconjugated rituximab and all conjugates stained only CD20-expressing CD19+ B cells strongly and specificity and no cross reactivity or increase in background staining was observed on CD3+ cells or other CD20-negative PBL (Figure 4 B and C and data not shown). The lowest yielding condition tested was the indirect conjugation method with high MSR, as we had expected that this method would be most likely to compromise binding and specificity as we assumed that loss of protein stability from increased crosslinker concentration and UC-1V150 loading would either reduce antibody binding or increase non-specific binding. Surprisingly however, we found that the lowest yielding conjugate tested (i.e. indirect method with high MSR) showed the closest level of binding to unconjugated rituximab,

better than other conjugates produced in conditions with better yield, suggesting that reduced protein yield does not necessarily indicate loss of antibody conformation or activity. None of the conjugates tested showed non-specific binding to any CD20-negative cells.

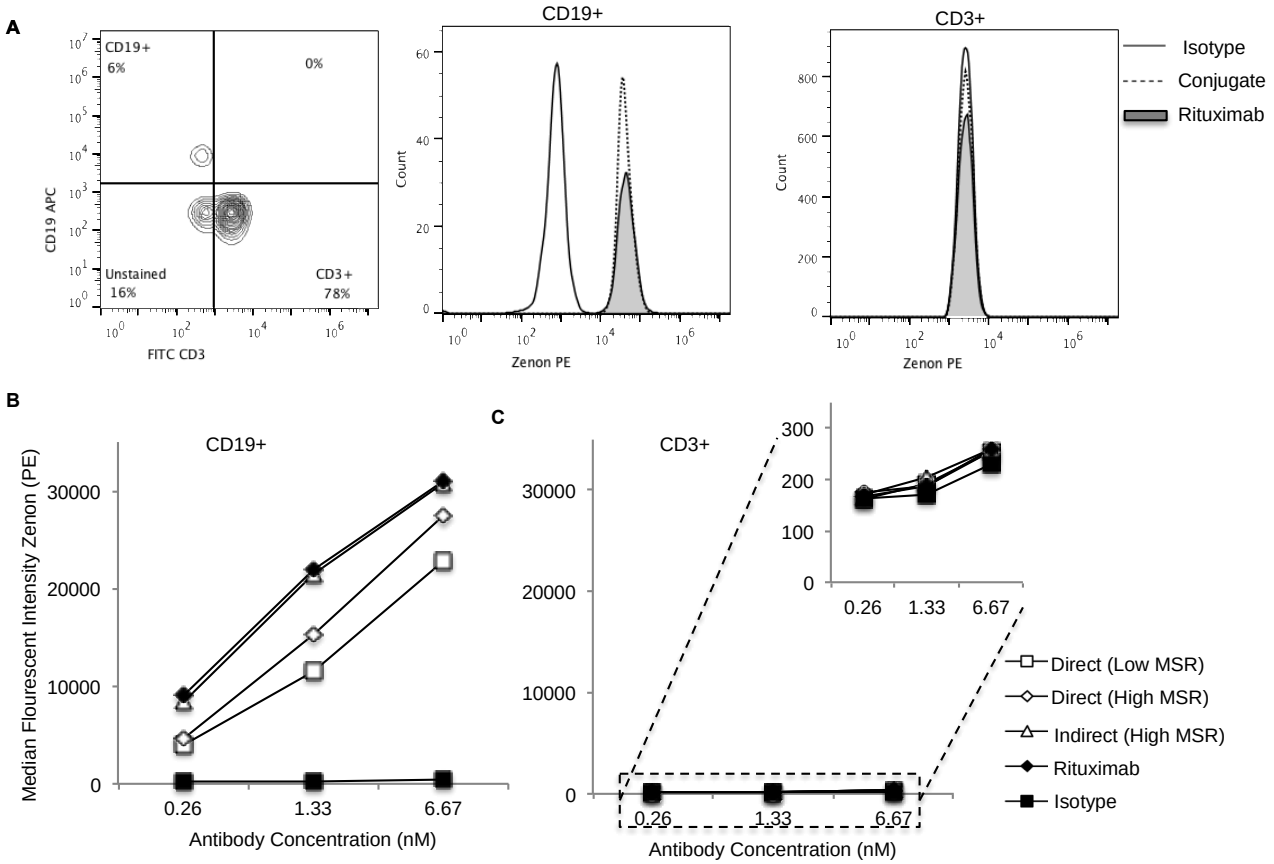

Figure 4 Antigen binding and specificity is maintained by rituximab UC-1V150 conjugates

Flow cytometric analysis of antibody and conjugate binding to human peripheral blood leukocytes was performed using Zenon™ labelling. **A** Binding of rituximab and conjugates of CD20-expressing B cells (CD19+) but not to CD20-negative T cells (CD3+) was observed. **B** Median fluorescent intensity of Zenon staining on gated CD20-expressing B cells (CD19+; left) and CD20-negative T cells (CD3+; right) was plotted for rituximab and various UC1V150 conjugates, and isotype control, at the indicated concentrations. Inset: equal background staining was observed on CD3+ cells for all antibodies and antibody conjugates tested.

Discussion

TLR7 and TLR8 agonists are potent proinflammatory small molecules with anti-viral and anti-tumor activity. However systemic TLR activation can lead to fatal toxic shock, and thus TLR agonists conjugated to macromolecules including proteins have been developed that localize inflammation and restrict systemic cytokine production. In order to determine if TLR agonists could be targeted specifically to tumors or antigen presenting cells using mAbs, we explored whether a small molecule TLR7 agonist could be covalently conjugated to the human IgG1 anti-CD20 mAb rituximab, used clinically to selectively kill B cells in non-Hodgkins lymphoma.

Successful targeting using ADC is dependent on an appropriate and effective conjugation method. A previously reported conjugation method referred to here as an indirect conjugation method (Figure 1) demonstrated that protein-TLR7 agonist conjugates could be constructed. However, we found that a simple modification to the conjugation method (Figure 1) increased compatibility to a wider range of proteins by reducing precipitation and thereby increasing conjugate yield for the glycoprotein IgG. We believe that protein loss during the indirect method occurs because the intermediate step is a labeled protein with pendent hydrazine groups which is then introduced into an acidic pH. Sugars have the ability to undergo mutarotation in acidic condition, exposing the normally cyclized aldehyde or ketone. At this point cross-linking can occur. Modulation of the molecular abundance of hydrazine containing linker used to label IgG help to illustrate this issue. No change in yield was seen when UC-1V150 concentration was varied but linker concentration kept constant (data not shown). The reaction of hydrazines with sugars is a well-known and documented process typically used for the labeling of glycoproteins in the presence of oxidizing agents [47,48]. The modified conjugation method proposed removes the possibility of cross-linking. It also improved the control and scalability of substitution ratio with minimal effect on yield (Figure 2 C) and reduced the reaction time to 2 h. This method also minimizes protein manipulation steps, highly beneficial given the extremely high cost of many recombinant or purified proteins in developmental vaccines and also experimental mAbs. Conjugation of 100 µg protein or less was achieved with the direct conjugation method (data not shown). Indeed, using the previously published indirect conjugation method, the low yields produced with higher substitution ratios gave insufficient conjugate to fully characterise *in vitro,* in contrast to the direct method.
For rapid evaluation of vaccine or tumor immunotherapy conjugates to TLR agonists, a simple method of quantifying conjugation efficiency is vital. A benefit of the bifunctional linker chosen is that it is UV active, allowing quantification of conjugates using UV spectroscopy. However, UV absorbance and extinction coefficients alter after conjugation through the hydrazine (Figure 1C), and the direct conjugation method therefore offers the added advantage that the UV active compound is reacted with TLR7 agonist drug prior to protein conjugation, and this reaction was monitored precisely to completion using NMR. In contrast, the indirect labelling method assumes that for every molecule of linker containing the UV active component there is a corresponding molecule of UC-1V150. When the linker is reacted first with protein it is not possible to determine by UV alone if any residual unreacted linker is present on the protein. We therefore confirmed the molecular substitution ratio using mass spectroscopy, which correlated very well with UV quantitation for all conjugates (Tables 2 and S1; Figures 2D and S1).

Previous reports have shown that conjugation to proteins and polymers can increase the potency of TLR agonists [28,49], and this was confirmed with IgG as the macromolecular scaffold. The influence of substitution ratio on pro-inflammatory activity has not been reported. From our studies it appears that the substitution ratio had relatively minimal effect on cytokine response compared to the initial effect of protein conjugation. Although when cytokine production was plotted against molar antibody concentration an increase in specific activity was observed with increasing substitution ratio, when plotted against the final concentration of UC-1V150 no significant difference in specific activity of cytokine production remained (Figure 3). This suggests that although protein conjugation increases

potency ~10-fold over UC-1V150 alone, the ratio of conjugation has little further effect on potency. The impact of TLR7 activation on anti-tumor antibody efficacy after conjugation remains to be established. Anti-tumour mAbs have range of effects including direct cytotoxicity, antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP). Since activation of TLR is expected to directly influence many of these processes, and the subsequent production of proinflammatory cytokines is likely to have further indirect effects on the efficacy of anti-tumor mAbs. Likewise, targeting of TLR7 agonists to subsets of DC for vaccine applications is likely to have many complex effects. Significant research is now required to fully understand the activity of antibody-TLR7-agonist conjugates. Current and planned studies focus on evaluating the ADCC and ADCP activity of these conjugates both *in vitro* and *in vivo*

For any ADC it is essential that the conjugation process avoids any loss of antibody function, especially antigen binding and specificity. Unsurprisingly, UC-1V150 conjugates showed a small reduction in binding for CD20, which we assumed was due to the use of a non-specific amine reactive conjugation method. This method can potentially label lysine residues in the FAB region leading to steric blocking of antigen binding or modulated on and off rates. Interestingly, conjugates produced by the indirect method with the highest substitution ratio show the least reduction in CD20 binding similar to that of native rituximab, which contrasts to the reduced yield with this method. Further structural and cell free antigen binding studies (e.g. surface plasmon resonance) are required to confirm this apparent difference between conjugation methods has a significant impact on antibody integrity and binding characteristics. Further *in vivo* targeting studies are also required to confirm specificity of conjugates. In future, site-directed conjugation methods could be developed that would avoid nonspecific amine targeting.

Previous studies conjugating vaccine antigens to TLR agonists have evaluated the impact of conjugation on protein integrity. Covalent conjugation of small molecule TLR agonists to HIV GAG protein has been shown previously to elicit a broad-spectrum adaptive immune response [50], as well as using small peptide sequences covalently conjugated to TLR agonists [51], suggesting that TLR conjugation does not significantly compromise protein structure and antigenicity. Recent data suggest that even under high labeling ratios important structural epitopes remain intact [52]. The presence of a lysine residue in the epitope determined if conjugation could occur at that site, if conjugation did occur, antibodies for that epitope had a reduced binding affinity, but importantly other epitopes remained accessible indicating limited global structural changes after conjugation [52]. Thus although the conjugation of TLR agonists to proteins using free amine reactive linkers lacks site specificity, the likelihood of structural changes or epitope masking is not expected to be a major problem. Indeed, amine-reactive conjugation to mAbs has been widely used as an effective and established strategy for ADC [53]

Conclusion

We demonstrate for the first time that small molecule TLR agonists can be coupled to a therapeutic mAb whilst retaining antigen binding, specificity, and TLR stimulatory activity. Previous methods for conjugation UC-1V150 to proteins were modified to improve compatibility with glycoproteins such as

therapeutic mAbs. The direct conjugation method improves protein yield, but also reduces protein handling times and is therefore ideal for rapid screening of different therapeutic antibodies. We confirmed UV quantification accurately correlated with mass increase again allowing rapid and high throughput analysis of conjugates. Using mAbs as a macromolecular scaffold to limit systemic TLR activation, combined with tumor or APC targeting, and enhanced TLR-stimulatory potency following protein conjugation, overall offers great potential in three distinct therapeutic areas. Tumor immunotherapy can be improved either by direct therapeutic targeting of TLR activation to the tumor, or by indirectly improving cytotoxicity of anti-tumor antibodies, and finally improved vaccine adjuvants can now be developed by targeting TLR activation to selected APC subsets.

Experimental Section

Materials

Empty spin columns and Nunc® Maxisorp ELISA plates were purchased from Fisher scientific (Loughbrough, UK). Spehadex™ G50 superfine was purchased from GE Healthcare (UK). PBS, FBS, RPMI and Zenon™ PE were purchased from Life Technology (Paisley, UK). 2-mercaptoethanol, EDTA, Bradford reagent, NaN_3, Streptavidin alkaline phosphatase conjugate, Sigmafast pNPP and Human IgG1 kappa isotype control were all purchased from Sigma Aldrich (Gilford, UK). *N*-(2,5-dioxopyrrolidin-1-yl)-6-(2-(propan-2-ylidene)hydrazinyl)nicotinamide was purchased from Solulink (San Diago, USA). Red cell lysis buffer, staining buffer, fixing buffer, ELISA and flow cytometer antibodies were purchased from eBioscience (Hatfield, UK). Cellstar 96 well tissue culture plates were purchased from Greiner (Stonehouse, UK). LAL chromogenic endpoint assay was purchased from Hycult biotech (Uden, Netherlands). Rituximab (MabThera, Roche) was obtained from the Royal Berkshire Hospital (Reading, UK).

Synthesis of 4-((6-amino-2-(2-methoxyethoxy)-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)benzaldehyde: UC-1V150

Compound 2, UC-1V150 was synthesized according to previously reported procedure[28] and retrieved as an off-white solid.

^{1}H NMR – ($(CD_3)_2SO$, 400 MHz): δ 10.06 (1H, s), 9.98 (1H, s), 7.88 (2H, d, *J* 8.4), 7.49 (2H, d, *J* 8.4), 6.58 (2H, s), 4.97 (2H, s), 4.27 (2H, t, *J* 4.4), 3.58 (2H, t, *J* 4.4), 3.26 (3H, s).

^{13}C NMR ($(CD_3)_2SO$, 100 MHz): δ 192.6, 159.7, 152.3, 149.1, 147.8, 143.8, 135.3, 132.5, 129.8, 128.2, 127.9, 98.59, 70.17, 65.2, 58.0, 42.2

HRMS (ESI) calculated for $C_{16}H_{18}N_5O_4^+$ (MH^+) 344.1353. Found 344.1353

N-(2,5-dioxopyrrolidin-1-yl)-6-(2-(propan-2-ylidene)hydrazinyl)nicotinamide **1**

^{1}H NMR ($(CD_3)_2SO$, 400 MHz): δ 10.39 (1H, s), 8.77 (1H, d *J* 2.4), 8.12 (1H, dd *J* 8.8, 2.4), 7.18 (1H, d *J* 8.8), 2.89 (4H, s), 2.01 (3H, s), 1.99 (3H, s)

Synthesis of 2,5-dioxopyrrolidin-1-yl (*E*)-6-(2-(4-((6-amino-2-(2-methoxyethoxy)-8-oxo-7,8-dihydro-9*H*-purin-9-yl)methyl)benzylidene)hydrazinyl)nicotinate **3**

N-(2,5-dioxopyrrolidin-1-yl)-6-(2-(propan-2-ylidene)hydrazinyl)nicotinamide (1 mg, $3.5x10^{-6}$ mol) was dissolved in $(CD_3)_2SO$ (450 µL) with UC-1V150 (1.2 mg, $3.5x10^{-6}$ mol) and $PhNH_2$ (5 mMol final conc, 200 µg). The mixture was monitored by NMR until the depletion of the peak at δ 9.98 and appearance of δ 11.83. The compound was used without further purification.

^{1}H NMR (crude) ($(CD_3)_2SO$, 400 MHz): δ 11.83 (1H, s), 10.08 (1H, s), 8.81 (1H, d, *J* 2.4), 8.20 (1H, dd, *J* 8.8, 2.3), 8.16 (1H, s), 7.71 (2H, d, *J* 8.2) 7.37 (d, 2H, J = 8.2), 6.97 (1H, d, *J* 7.7), 6.54 (2H, s), 4.90 (2H, s), 4.27 (2H, t, J = 4.7), 3.59, (2H, t, J = 4.7), 3.28 (s, 3H), 2.89 (s, 4H).

^{13}C NMR ($(CD_3)_2SO$, 175 MHz): 179.7, 170.7, 160.9, 159.9, 152.3, 151.9, 149.1, 147.8, 143.3, 142.9, 139.2, 138.7, 133.7, 132.6, 128.0, 126.9, 110.8, 98.5, 70.3, 65.4, 58.1, 42.3, 30.7, 29.6, 27.4, 25.5

NMR and mass spectra are reported in supplementary data.

Conjugation of 2,5-dioxopyrrolidin-1-yl (*E*)-6-(2-(4-((6-amino-2-(2-methoxyethoxy)-8-oxo-7,8-dihydro-9*H*-purin-9-yl)methyl)benzylidene)hydrazinyl)nicotinate to rituximab.

Preparation and equilibration of size exclusion spin columns

Sephadex® G50 superfine (1 g) was added to PBS (20 mL, varying pH) and allowed to hydrate for at least 4 h, typically overnight. The supernatant was removed and a further 1 mL of PBS was added and mixed to allow manipulation by pipette. 700 µL of the solution was transferred to a spin column, which was placed in a standard 1.5 mL eppendorf and centrifuged at 200 G for 1 minute. A further 100 µL of PBS was loaded onto the Sephadex bed then centrifuged for 1 minute at 200 G. A further 100 µL aliquot was loaded and centrifuged for 2 minutes at 200 G. The spin column was transferred into a new eppendorf and used subsequently. The efficiency of removal of UC-1V150 and bifunctional linker from mAb with these micro size exclusion columns was determined, and found that mAb eluted exclusively in the column void volume, fraction 1. Residual DMSO was not observed until fraction 3 and that no trace of unconjugated small molecules eluted in fractions 1-5 when using 100 µL eluent indicating efficient separation of mAb and unconjugated active compound.

Direct conjugation of UC-1V150 to rituximab to produce TLR7 agonist-rituximab conjugates

Rituximab (100 µg, 6.6×10^{-10} mol) was desalted in to PBS (pH 9) using a pre-equilibrated size exclusion spin column. The solution was briefly vortexed before adding a 10x molar excess of **3** (1 µL) and vortexed before incubating at room temperature for 2 h. To modulate the labeling ratio, eg to achieve a 2:1 TLR agonist-rituximab conjugate a 20x molar excess of **3** (2 µL) was added. The mixture was vortexed before desalting as previously described into PBS (pH 7.2) for subsequent characterisation.

Indirect conjugation of NHS:UC-1V150 to achieve a 1:1 TLR7 agonist – rituximab conjugate

Rituximab (100 µg, 6.6×10^{-10} mol) was desalted in to PBS (pH 9) using a pre-equilibrated size exclusion spin column. The solution was briefly vortexed before adding a 2x molar excess of **(2)** predissolved in DMSO to a final concentration 1 mg/mL. The mixture was incubated for 2 h at room temperature before vortexing and desalting into PBS (pH 6). The solution was vortexed and an 8x molar excess of UC-1V150 predissolved in DMSO (1 mg/mL) was added mixed and incubated at room temperature for a minimum of 12 h. The solution was vortexed before desalting as previously described into PBS (pH 7.2) for subsequent characterization.

Assessment of TLR7 agonist loading ratio

To calculate the loading ratio of TLR 7 agonist on rituximab, first the concentration of nicotinate **3** in the sample was calculated by A_{340} then divided by the total protein concentration determined by Bradford assay. UV/vis absorbance spectrum was recorded between the wavelength of 200-700 nm at 5 nm increments on a Biotek Take 3 system (Potton, UK). Absorbance readings were corrected to a

pathlength of 1 cm using internal path length determination. A standard curve was produced by serial dilution of **3** and A_{340} plotted to produce a formula y = 0.0038x - 0.0182. A UV/Vis spectrum of conjugates was recorded and the formula used to determine TLR agonist concentration in the conjugate. Since **3** is UV active and has λ_{max} at 274 and 340 nm, A_{280} cannot be used to determine protein concentration. A Bradford assay was therefore used according to the manufactures instructions for 96 well assays (Sigma Aldrich). The mass of rituximab and the subsequent mass increase of the conjugates was monitored using a Brukner Ultraflex MALDI TOF/TOF mass spectrometer in high range mode, 25 kV 350 ns ion pulse with BSA as a calibration (Table S1 and Figure S1).

Endotoxin contamination

Endotoxin is a component of bacterial cell walls with potent inflammatory response through TLR4, and is also a common laboratory contaminant. Synthesis and conjugation was completed in sterile environment and only endotoxin free reagents used. To confirm the absence of endotoxin contamination, a LAL assay was used to quantify the amount of soluble endotoxin in all reagents. Endotoxin quantification was performed according to manufacturer's instruction (Hycult, Netherlands), and all conjugates tested were below the limit of detection of 0.08 EU/mL.

Stimulation of RAW264.7 macrophages with rituximab-TLR7 agonist conjugates

RAW cells were maintained and subcultured as described in ATCC procedures for this cell line. Stimulation assays were performed in tissue culture grade 96 well plates. Conjugates were diluted serially 1:2 (100 µL final volume) in complete media (RPMI 1640, 10 % FBS, 0.05 mM 2-ME) then $2x10^6$ cells/mL freshly passaged cells were added in 100 µL to give a final volume of 200 µL. Plates were incubated for 24 h before the analysis of supernatants for pro-inflammatory cytokines. IL-12p40 concentrations in supernatants of RAW 264.7 stimulation assays were determined by sandwich ELISA following a standard sandwich ELISA protocol. Nunc maxisorp 96 well ELISA plates were coated overnight with IL-12p40 capture antibody (C15.6) at 2 µg/mL in carbonate buffer (pH 9.6, 50 µL per well), followed by 2 h with 200 µL per well block buffer (PBS, 2.5 v/v% FBS, 0.02 w/v% NaN_3). 100 µL per well detection antibody (C17.8) at 1 µg/mL in block buffer was added. Stimulated RAW264.7 supernatants were diluted 1:7.5 in block buffer, and compared to 1:2 dilution of recombinant IL-12p40 standards with a top concentration of 10 ng/mL in block buffer, with 150 µL sample per well. Extravidin alkaline phosphatase conjugate was prepared 3:10,000 dilution in PBS. Sigmafast pNPP substrate was prepared according to manufactures instructions.

Determination of specific binding to human CD20 in human peripheral blood leukocytes

Peripheral blood from healthy donors was collected in lithium heparin vacutainers. Erythrocytes were lysed by the addition of red cell lysis buffer and incubation at room temperature for 10 minutes before centrifuging at 200 G for 5 minutes. The supernatant was removed and the pellet resuspended in red cell lysis buffer. After washing, PBL were resuspended in staining buffer at $1x10^7$ cells/mL. Zenon™ PE human FC labeling kit was used to fluorescently label rituximab, rituximab-UC-1V150 conjugates and an isotype control following manufactures guidelines (Life Technologies). Cells were first stained with APC-anti-human-CD19 and FITC anti-human-CD3, and subsequently Zenon-labelled antibodies and conjugates were added and incubated for 30 mins on ice before fixing in fixation buffer. Fixed samples were analyzed on a BD Accuri™ C6 flow cytometer (Oxford, UK) and data analysed using FlowJo (Ashland, USA).

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