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Research article

Investigating the affinity of poly *tert*-butyl acrylate toward Toll-Like Receptor 2

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Abstract: Despite the high safety profile of peptide-based vaccines over conventional counterparts, the inability of small peptides to produce a strong immune response represents the main obstacle for the development of these types of vaccines. Introducing a self-adjuvanting moiety such as poly *tert*-butyl acrylate can overcome this problem. However, the mode of action of this polymer to produce the desired humoral and/or cellular immune response is still unknown. An AlphaScreen assay along with the cell-free expression technique were employed to evaluate the affinity of this polymer toward toll-like receptor 2 (TLR2) for stimulation of innate immunity. In this study, B-cell epitope, J14, derived from the M protein of group A streptococcus (GAS) was used in conjugation with the poly *tert*-butyl acrylate as well as a biotin moiety. Pam2Cys analogue, the potent TLR2 agonist, was synthesized and used as a positive control in this work. The AlphaScreen assay showed the inability of polymer to bind to TLR2, while the Pam2Cys displayed very strong binding to TLR2 as expected. This result indicated that poly *tert*-butyl acrylate does not express its immunogenic effects through recognition by TLR2 and therefore further studies are required to determine its mode of action.

Keywords: TLR2 agonists; poly *tert*-butyl acrylate; vaccines; AlphaScreen assay; adjuvant

1. Introduction

Peptide-based vaccines can be used to avoid the drawbacks of conventional or protein-based vaccines. These drawbacks include toxicity, allergy and autoimmune responses triggered by immunization [1]. For example, using the whole pathogen group A streptococcus (GAS) or its membrane protein, M protein, as an antigen in vaccine development can cause rheumatic heart disease due to the similarity of the antigen and heart protein [2,3]. The use of a small M protein-derived peptide sequence, J14 epitope, as an antigen in peptide-based vaccine did not cause this autoimmune complication [4]. However, peptide alone cannot produce a strong immune response. Therefore, incorporating an immunestimulant compound, adjuvant, with the peptide is essential to obtain the desired immune response against the antigen. The toxicity and low number of available licensed adjuvants directed the attention of scientists to find new ways to overcome this problem. In our laboratory, poly *tert*-butyl acrylate was used for the first time as a self-adjuvanting molecule in a chemical conjugation with antigens [5]. We proved the ability of this polymer to trigger the humoral immune system to produce IgG antibodies and to activate cellular immunity through the activation of $CD8^+$ T cells. For example, a series of poly *tert*-butyl acrylate-J14 vaccine candidates were able to induce strong antibody production [6,7], which were able to opsonize GAS [8]. Also, poly *tert*-butyl acrylate conjugated with $E7_{44–57}$ epitope, derived from the human papilloma virus (HPV) 16 E7 protein, displayed a high efficacy as a therapeutic vaccine to eradicate tumor cells *in vivo* [9–12].

Toll-like receptors (TLRs) play an important role to link the innate and adaptive immunity as the activation of these transmembrane proteins leads to stimulation of both humoral and cellular immunity [13–15]. TLR2 is an important receptor that can be targeted by many microbial components such as lipopeptides, lipoarabinomannans, lipomannans, glycosylphosphatidylinositol, lipoteichoic acid, and a range of proteins including lipoproteins and glycoproteins, zymosan and peptidoglycan (56–60 kDa) [16–20]. Mixing or direct conjugation of synthetic TLR2 agonists with antigens produced strong adjuvant activity [21,22].

Improving the capability of poly *tert*-butyl acrylate to elicit a stronger immune response requires well understanding of its mode of action. Poly *tert*-butyl acrylate 1 is similar in its hydrophobicity to TLR2 agonists such as Pam2Cys, therefore we assumed that this receptor might be involved in recognition of the polymer.

2. Materials and methods

Compounds 2 [23], 3 [10], and 4 [23], were synthesized as previously reported.

2.1. Synthesis of compound 1

A mixture of Poly *tert*-butyl acrylate (3) (3 mg, 0.2 μmol, 1 equiv.) and biotin-J14-azide 4 (1.97 mg, 0.4 μmol, 2 equiv.) was dissolved in DMF (1 ml), and a copper wire (60 mg) was added. The air in the reaction mixture was removed by nitrogen bubbling. The reaction mixture was covered and

protected from light with aluminum foil and stirred at 50 ℃ under nitrogen for 4 h. The wires were filtered off from the warm solution and washed with 1 ml of DMF. Millipore endotoxin-free water (7 ml) was slowly added to the solution (0.005 ml/min). Particles formed through the self-assembly process were exhaustively dialyzed against endotoxin-free water using presoaked and rinsed dialysis bags (Pierce Snakeskin, MWCO 3K). The yield of the reaction was 1.95 mg, 25%. The final formulation was self-assembled into particles in water with diameters 300–400 nm as observed by dynamic light scattering (DLS) using a Malvern Zetasizer Nano Series with DTS software. Size was analyzed using a noninvasive backscatter system. Multiplicate measurements were performed at 25 ℃ with a scattering angle of 173 ° using disposable cuvettes and the number-average hydrodynamic particle diameter was reported.

2.2. TLR expression

The open reading frame (ORF) of TLR2 was obtained from the Diamantina Institute, UQ. The extracellular domain of TLR2 was expressed with mCherry fusion tag in vector pCellFree G08 [23,24]. TLR2 was expressed using the *Leishmania tarentolae* extract prepared as previously described [25,26]. Protein was resolved on NuPage Novex 4%–12% SDS-PAGE gel (Invitrogen, Australia) and scanned for fluorescence using the ChemiDoc MP System (Bio-Rad, Australia). Preheating gel samples to 72 ℃ instead of > 95 ℃ allows the mCherry fluorescence to persist in the SDS-Page gel, allowing expression size to be validated against the ORF size expected. TLR2 fusion protein showed its expected size (91 kDa).

2.3. AlphaScreen proximity assay

The extracellular domain of TLR2 fusion protein was expressed in *L. tarentolae* extract at 27 ℃ for 30 minutes. 2.5 μl of protein expression reaction was then mixed with an equal volume of serially diluted biotin-labeled compound 1 ranging from 100 nM to 50 μM. Expression reaction refers to the *Leishmania tarentolae* cell-free protein synthesis reaction, which expresses the TLR fusion protein. Then, protein expression was continued by incubation at 27 ℃ for an additional 2.5 hours. The resulting mixture was diluted 4 fold in buffer A (25 mM HEPES, 5 mM NaCl), followed by 3 more 10-fold dilutions in buffer A. From each dilution, 2 μl was added to 12.5 μl of buffer B (0.32 μg/μl anti-cMyc acceptor beads, 25 mM HEPES, 50 mM NaCl, 0.001% (v/v) casein and 0.001% (v/v) Nonidet P-40), and incubated in the dark for 45 min to allow acceptor beads to couple with cMyc-tagged fusion protein. Subsequently, 0.4 μg of Streptavidin-coated donor beads in 2 μl of buffer A was then added to each dilution and incubated for 45 min in the dark. The AlphaScreen signal was measured with the Envision Multilabel plate reader (Perkin Elmer, Australia) according to the manufacturer's recommended settings (excitation: 680/30 nm for 0.18 s, emission: 570/100 nm after 37 ms). For each concentration of compound 1, the maximal AlphaScreen signal was determined and plotted against the sample dilution.

3. Results and discussion

Here, we performed the first attempt to investigate the ability of poly *tert*-butyl acrylate to bind with TLR2 via the AlphaScreen assay and cell-free expression techniques. Pam2Cys is a potent

TLR2 agonist that was used both *in vitro* and *in vivo* as a self adjuvanting moiety [27,28]. In this study, Pam2Cys was used as a positive control. Polymer 1 and its Pam2Cys analogue 2 were tagged with a biotin moiety (Figure 1). For the attachment of the polymer 3 to J14 epitope, the azide derivative of biotin-J14 (4-biotin-KQAEDKVKASREAKKQVEKALEQLEDKVKGK($OCCH_2N_3$)G) was synthesized by using solid phase peptide synthesis (SPPS) [23]. Compound 4 was then conjugated with the alkyne group on polymer 3 via the copper-catalyzed azide-alkyne cycloaddition (CuAAC) reaction to give the final compound 1 in 25% yield (Figure 2). The particle size diameter, 300–400 nm, of the final formulation after self-assembly in water was measured by dynamic light scattering (DLS).

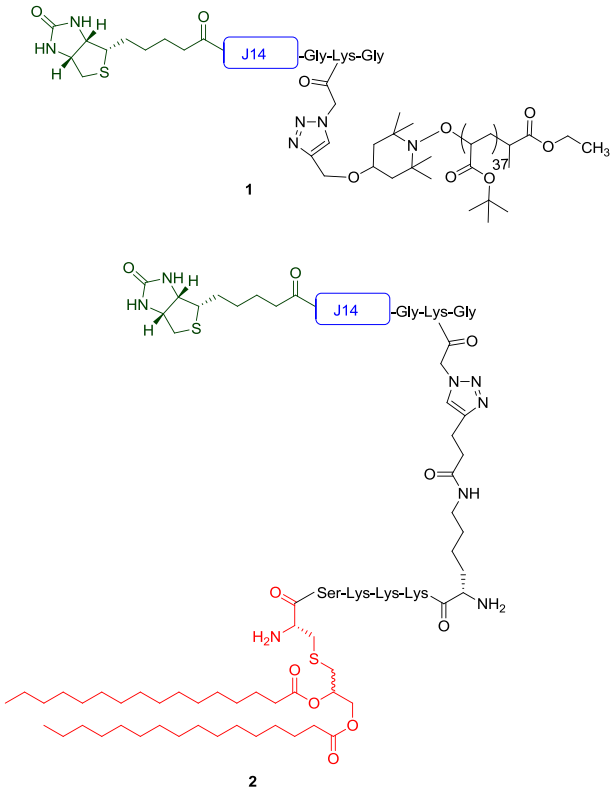

Figure 1. Structures of poly *tert*-butyl acrylate-J14-biotin (1) and Pam2Cys-J14-biotin (2).

The AlphaScreen assay was performed to test the affinity of compounds 1 and 2 toward the *in vitro* cell-free expressed extracellular domain of TLR2 [23]. The biotin tagged compounds 1 and 2 were bound to a streptavidin-coated donor bead. Anti-cMyc acceptor beads recruited the TLR2 through its cMyc tag. Upon excitation, the donor bead generated singlet oxygen with a half-life of 4 μs and diffusive distance of ~200 nm. Interaction of the compounds (1 and 2) with TLR2 brought donor and acceptor beads into close proximity, which allowed the singlet oxygen to react with thioxene derivatives of the acceptor beads. This is turn stimulates luminescence which is detected as an AlphaScreen signal in counts per second (cps). As expected, the Pam2Cys analogue bound to TLR2 (Figure 3). In contrast, polymer 1 did not show any affinity to bind to TLR2 (Figure 3). This demonstrated that the ability of conjugate 1 to stimulate an immune response occurs through a different mechanism rather than binding to TLR2.

4. Conclusion

Finding the mode of action of poly *tert*-butyl acrylate may help in modulating the immunogenic activity of the polymer. Two biotin conjugates 1 and 2 were successfully designed and synthesized to investigate the affinity of poly *tert*-butyl acrylate toward TLR2. The extracellular domain of TLR2 was successfully expressed by using the cell-free expression technique. The well-known TLR2 agonist Pam2Cys 2 demonstrated a very strong binding to TLR2; however, the polymer analogue 1 did not show any affinity by applying the AlphaScreen assay. Discovery of the mechanism of action of poly *tert*-butyl acrylate is still under progress.

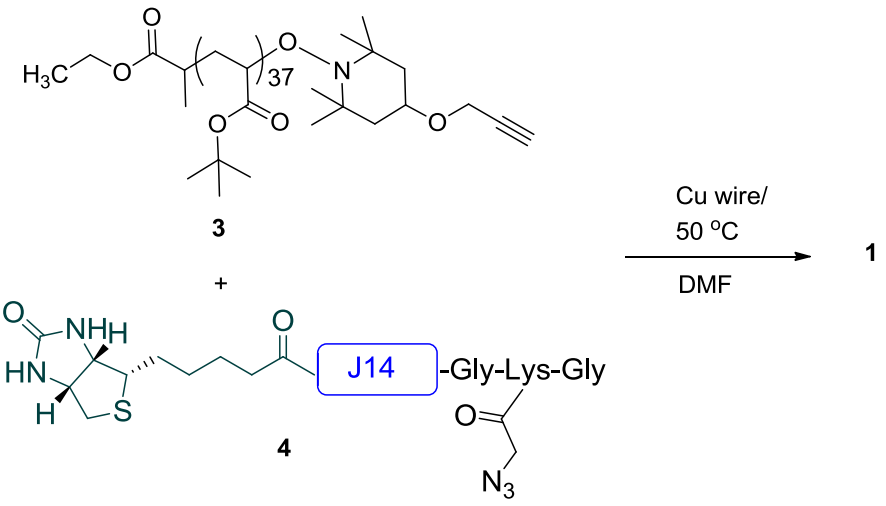

Figure 2. Synthesis of 1 through CuAAc reaction between the alkyne derivative of poly *tert*-butyl acrylate (3) and azide derivative of J14-biotin (4) in presence of copper wire and DMF at 50 ℃.

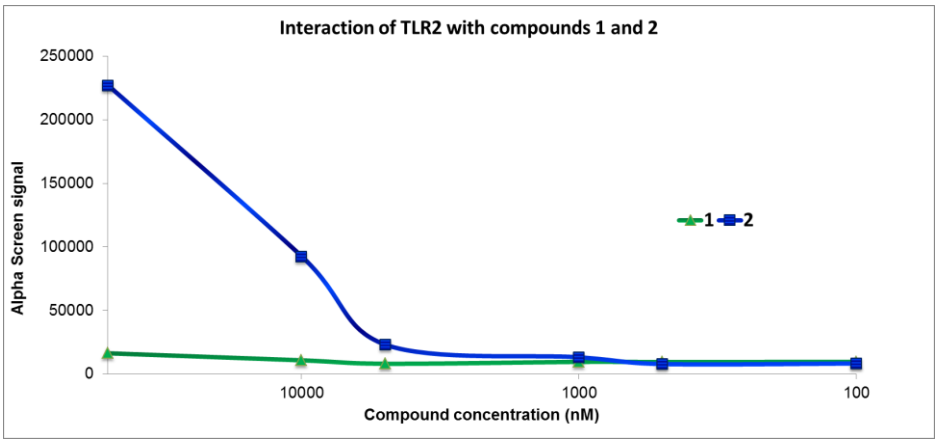

Figure 3. Analysis of interactions of conjugates 1 and 2 with *in vitro* expressed TLR2 using the AlphaScreen proximity assay. The AlphaScreen assay was performed at 10 nM concentration of cell-free expressed TLR2, while varying the concentrations (100 nM–50 μM) of 1 or 2.

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Conflict of interest

All authors declare that they have no conflict of interest in this paper.

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