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**14. ABSTRACT**

Immune checkpoint inhibitors (ICI) have revolutionized cancer treatment by restoring the ability of T cells to recognize and destroy tumor cells, resulting in an unprecedented anti-cancer immune response and efficacy. However, despite their remarkable success in treating many malignancies, not all tumors fully respond to ICI monotherapy due to their poorly immunogenic nature, low T cell infiltration into the tumor bed, and insufficient T cell activation. Efficacy may also be limited by the low tumor penetration of monoclonal antibodies (mAbs) —the tumor-targeting ICIs of choice— due to their large size and structural inflexibility. To further activate T cells, combination therapies with cytokines, such as interleukin-15 (IL-15) and IL-2 (aldesleukin), have been utilized. Unfortunately, these treatments result in life-threatening systemic toxicity and ambiguous clinical efficacy due to their non-selective nature. Furthermore, even if these problems are solved, the short plasma half-life (~1.4 h) of IL-2 and IL-15 limits their therapeutic utility. PEGylation —the conjugation of PEG to biologics— is commonly used to overcome this limitation. Unfortunately, PEG is antigenic, which has caused severe allergic reactions in some patients and has led to the withdrawal of several PEGylated drugs from the market and termination of Phase III clinical trial of a PEGylated drug candidate. These problems have been traced to pre-existing PEG antibodies found in ~50% of the U.S. population and even in individuals who have not previously received a PEGylated drug. Hence, there is an unmet need for immunotherapy that provides high anti-cancer efficacy and overcomes the low tumor penetration of mAbs and the toxicity, lack of selectivity, and pharmacokinetic (PK) limitations of cytokines using technologies beyond PEGylation. Here, we developed hyperbranched PEG-like polymer conjugates of a selective anti-cancer cytokine and an immune checkpoint inhibitor for cancer immunotherapy. The conjugates showed high binding affinity to their endogenous receptors and had high in vitro activity. The cytokine conjugates showed comparable in vivo anti-cancer efficacy to the PEG-attached conjugate in colon cancer model of mice.

**15. SUBJECT TERMS**

Polyethylene glycol, cancer immunotherapy, immune checkpoint inhibitor, interleukin-2, PEG immunogenicity, programmed death receptor ligand, PD-L1, stealth polymer, bioconjugate

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## 1. INTRODUCTION:

Immune checkpoint inhibitors (ICI) have revolutionized cancer treatment by restoring the ability of T cells to recognize and destroy tumor cells, resulting in an unprecedented anti-cancer immune response and efficacy.[1-3] However, despite their remarkable success in treating a number of malignancies, not all tumors fully respond to ICI monotherapy due to their poorly immunogenic nature, low T cell infiltration into the tumor bed and insufficient T cell activation.[1,2] Efficacy may be also limited by the low tumor penetration of monoclonal antibodies (mAbs) —the tumor-targeting ICIs of choice— due to their large size and structural inflexibility.[4,5] To further activate T cells, combination therapies with cytokines, such as interleukin-15 (IL-15)[6-8] and IL-2 (aldesleukin), have been utilized.[1,9-13] Unfortunately, these treatments result in life-threatening systemic toxicity and ambiguous clinical efficacy due to their non-selective nature.[9,12,15,16] Even if these problems are solved, the short plasma half-life (~1.4 h) of IL-2 and IL-15 limits their therapeutic utility.[8,9] PEGylation —the conjugation of PEG to biologics— is commonly used to overcome this limitation.[17] Unfortunately, PEG is antigenic,[27-30] which has caused severe allergic reactions in some patients[18,30,31] and has led to the withdrawal of several PEGylated drugs from the market and termination of Phase III clinical trial of a PEGylated drug candidate. These problems have been traced to pre-existing PEG antibodies that are found in ~50% of U.S. population[18,22] and even in individuals who have not previously received a PEGylated drug.[27,29,31,32] Hence, there is an unmet need for an immunotherapy that provides high anti-cancer efficacy and overcomes the low tumor penetration of mAbs and the toxicity, lack of selectivity and pharmacokinetic (PK) limitations of cytokines using technologies beyond PEGylation. The **objective** of this research is to develop long-acting PEG-like conjugates of a modular, oligomeric inhibitor of programmed death receptor ligand (PD-L1) and a selective, co-stimulatory cytokine —Neo 2/15— for combination immunotherapy. This research is based on the **overall hypothesis** that we can: **(1)** solve the low tumor penetration problems of mAb-based ICIs with a modular, selective, high affinity and small protein therapeutic—PD-L1 binding protein (PD-L1BP)— and **(2)** address the selectivity, toxicity, suboptimal PK and PEG antigenicity problems of cytokines with a potent, selective and co-stimulatory cytokine —Neo 2/15— by conjugation with the "next gen" PEG —POEGMA— that does not bind to pre-existing PEG antibodies and improves the PK of the biologic drugs.

## 2. KEYWORDS:

Polyethylene glycol, cancer immunotherapy, immune checkpoint inhibitor, cytokine, interleukin-2, PEG immunogenicity, programmed death receptor ligand, PD-L1, stealth polymer, bioconjugate

## 3. ACCOMPLISHMENTS:

**What were the major goals of the project?**

**Task 1. Identify the optimal POEGMA $M_w$ that best balances PK, binding affinity, and pharmalogical activity.**

~~Subtask 1.1. Obtain IACUC~~ and ACURO approval (1-5 months; 100% complete)

Subtask 1.2. PD-L1BP and Neo 2/15 expression (1-6 months; 100% complete)

Subtask 1.3. Synthesis, purification and characterization of POEGMAs (1-6 months; 100% complete)

Subtask 1.4. Synthesis, purification and physical characterization of PDL1BP-POEGMA and Neo 2/15-POEGMA conjugates (3-9 months; 75% complete)

Subtask 1.5. Determine binding affinity of PD-L1BP-POEGMA and Neo 2/15-POEGMA conjugates (6-9 months; 75% complete)

Subtask 1.6. Determine pharmacological activity of PD-L1BP-POEGMA and Neo 2/15-POEGMA conjugates (6-9 months; 75% complete)

Subtask 1.7. Determine PK of PD-L1BP-POEGMA and Neo 2/15-POEGMA conjugates (9-12 months; 0% complete)

**Task 2. Assess anti-cancer efficacy of the optimized conjugates as a single-agent and combination treatment and investigate their PEG antibody reactivity**

Subtask 2.1. Obtain IRB and HRPO approval for use of plasma samples (1-5 months; 100% complete)

Subtask 2.2. Mid-scale synthesis, purification and physical characterization of PD-L1BP and Neo 2/15 conjugates for animal studies (12-24 months; 100% complete)

Subtask 2.3. Determine optimal number of cleavage sites and location on PD-L1BP-POEGMA (12-15 months; 0% complete)

Subtask 2.4. Determine optimal dose for PD-L1BP-POEGMAopt (15-18 months; 0% complete)

Subtask 2.5. Determine optimal dose for Neo 2/15-POEGMAopt (15-18 months; 0% complete)

Subtask 2.6. Assess anti-cancer efficacy of PD-L1BP-POEGMAopt and Neo 2/15-POEGMAopt as single-agent and combination therapy (18-21 months; 50% complete)

Subtask 2.7. Assess anti-cancer efficacy of PD-L1BP-POEGMAopt and Neo 2/15-POEGMAopt in conjunction with standard-of-care chemotherapy (21-24 months; 0% complete)

Subtask 2.8. Determine reactivity of PD-L1BP-POEGMAopt and Neo 2/15-POEGMAopt towards patient-derived PEG antibodies (15-24 months; 0% complete)

**What was accomplished under these goals?**

**Preliminary Data on Aim 1**

***Neo 2/15 and its site-specific and stoichiometric POEGMA conjugates have high affinity, selectivity, and pharmacological activity.*** Neo 2/15 is a *de novo* designed and potent mimic of IL-2 and IL-15, that selectively stimulates murine and human cytotoxic T and natural killer (NK) cells by binding the β and ***γ*** subunits of IL-2 and IL-15 receptors (IL-2/15Rβ***γ***), which are shared between IL-2R and IL-15R. Neo 2/15 eliminates the selectivity and toxicity concerns of its native counterparts because it does not react with IL-2Rα or IL-15Rα, which are expressed on many other cell types, including immune-suppressive regulatory T ($T_{reg}$) and endothelial cells.[25] Neo 2/15 monotherapy has shown higher efficacy than native IL-2 in treating colorectal cancer in mice without inducing $T_{reg}$ expansion.[25]

To synthesize Neo 2/15-POEGMA conjugates, we first synthesized gram scale amounts of monodisperse and azide-functional POEGMAs at varying weight-averaged molecular weight ($M_w$) by atom transfer radical polymerization (ATRP), determined by SEC-Multiangle Light Scattering (SEC-MALS; data not shown). Briefly, triethylene glycol methyl ether methacrylate (EG3) monomer was passed through basic alumina column to remove inhibitors. A catalytic complex was prepared by mixing tris(2-pyridylmethyl) amine (TPMA) and copper (II) bromide ($CuBr_2$) in ultrapure water with 18.2 MOhm resistivity at a final concentration of 0.8 M and 0.1 M, respectively. In a typical copolymerization, a Schlenk flask contained EG3 (10 mmol), azide functional polymerization initiator (0.2 M in methanol; 250 µl; Sigma), the catalytic complex (125 µl), methanol (5.75 ml) and 100 mM NaCl (11.613 ml). The polymerization flask was sealed and cooled to 0$^{o}$C in an ice bath. A separate Schlenk flask contained 64 mM ascorbic acid in ultrapure water. Both flasks were purged with argon for 45 min on ice to remove oxygen. After deoxygenation, the ascorbic acid solution was continuously injected into the polymerization flask at a rate of 1 µl min$^{-1}$ using a syringe pump under an inert atmosphere for 30 minutes-2 hours. The resulting solution was kept under vacuum to remove methanol and freeze-dried overnight. The resulting POEGMA was dissolved in acetonitrile and passed through a neutral alumina column to remove the catalytic complex. POEGMA was purified from unreacted monomer by precipitation in cold diethyl ether, followed by overnight evaporation of excess diethyl ether under vacuum. The resulting polymers were monodisperse and had a varying $M_w$ between 17-100 kDa.

Having synthesized well-defined POEGMAs, we next expressed Neo 2/15. Neo 2/15 was cloned and expressed in *E. coli* with a C-terminus sortase-A recognition site (LPETG) and a polyhistidine tag, yielding Neo 2/15-LPETG-$His_6$. The LPETG peptide acts as the sortase ligation site for introduction of a reactive handle for click reaction, while polyhistidine tag enables purification via immobilized metal affinity chromatography (IMAC). We purified Neo 2/15-LPETG-$His_6$ from *E. coli* lysate using IMAC, followed by size-exclusion chromatography (SEC). The purity was visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 4-20% precast Tris-HCl gels (Bio-Rad), followed by staining with Simply Blue Safe Stain (Thermo Scientific) and gel densitometry analysis using Image Lab software (Bio-Rad). The Neo 2/15-LPETG-$His_6$ concentration was measured by UV–visible spectroscopy on an ND-1000 Nanodrop spectrophotometer (Thermo Scientific) using the known extinction coefficient of the protein. The resulting Neo 2/15-LPETG-$His_6$ had an expected $M_w$ of 12.9 kDa and ~96% purity **(Figure 1).**

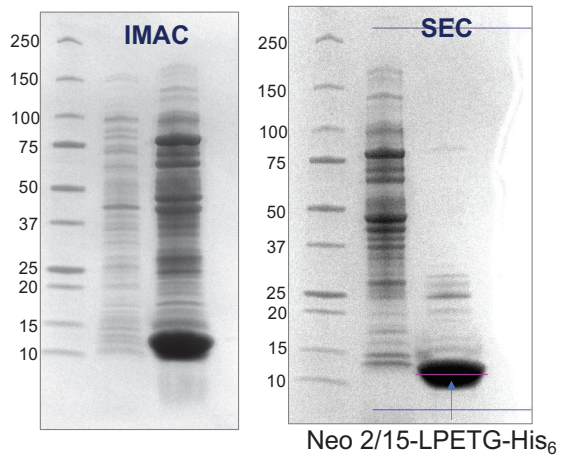



**Figure 1. SDS-PAGE analysis of Neo 2/15-LPETG-His$_6$.** IMAC and SEC purifications yield ~96% purity.

A bio-orthogonal DBCO group was installed on the C-terminus of Neo 2/15 by sortase A-mediated native peptide ligation, yielding Neo 2/15-DBCO. Briefly, Neo 2/15-LPETG-His$_6$ (100 μM) and His$_6$-Sortase A (50 μM) were reacted in the presence of triglycine-DBCO (Gly$_3$-DBCO) (5 mM; Click Chemistry Tools) in ligation buffer (50 mM Tris, 150 mM NaCl, and 10 mM $CaCl_2$; pH 7.5) at room temperature for 16 hours. Neo 2/15-DBCO was purified from the reaction mixture by reverse immobilized metal affinity chromatography using an AKTA Purifier (GE Healthcare) equipped with a photodiode array operating at 220 and 280 nm and a HisTrap HP (GE Healthcare) column. Neo 2/15-DBCO was collected in the flow-through as it was the only species that did not carry an oligohistidine tag, and hence did not bind to the resin. Neo 2/15-DBCO was concentrated by ultrafiltration using Centricon 70 (Millipore Sigma) filters with a 3,000 Da molecular weight cut-off (MWCO), followed by dialysis into cold water and lyophilization.

In preliminary studies, we determined that N-terminus of Neo 2/15 was critical to its function. To preserve its activity, we conjugated the POEGMAs to the C-terminus of Neo 2/15 using strained-alkyne azide bio-orthogonal click chemistry.[17-20] Neo 2/15-DBCO and azide-functional POEGMA were dissolved in PBS at a 1.05:1 ratio and reacted overnight at 4ºC. Neo 2/15-POEGMA conjugates were purified by a single round of anion exchange chromatography using an AKTA purifier equipped with a photodiode array detector operating at 220 and 280 nm and a HiTrap Q HP column (GE Healthcare) at 4ºC using 20 mM Tris-HCL as the binding buffer and 20 mM Tris-HCl + 1 M NaCl as elution buffer. The purified conjugates were concentrated by ultrafiltration using Amicon filters (Millipore Sigma) with a 3,000 Da MWCO, followed by dialysis into the water at 4ºC overnight and lyophilization. In addition to the POEGMA conjugates of Neo 2/15, its PEG conjugate was also synthesized using the same methodology and an azide-functional PEG (Creative PEGworks) with an $M_w$ of 40 kDa (data not shown). The resulting Neo 2/15 conjugates were monodisperse with a polydispersity index ($Đ$)<1.3 and had varying $M_w$, as measured by SEC-MALS **(Figure 2a)**. The conjugates had a larger hydrodynamic size ($R_h$) than unmodified Neo 2/15-DBCO and the renal excretion threshold (~3nm), which is defined as the size of serum albumin[18,26] **(Figure 2b; Table 1)**, as measured by dynamic light scattering (DLS). Neo 2/15-PEG had a larger $R_h$ than Neo 2/15-POEGMA at the same $M_w$, possibly because amorphous and linear PEG has a much larger $R_h$ than hyperbranched and compact POEGMA of the same $M_w$ (data not shown).

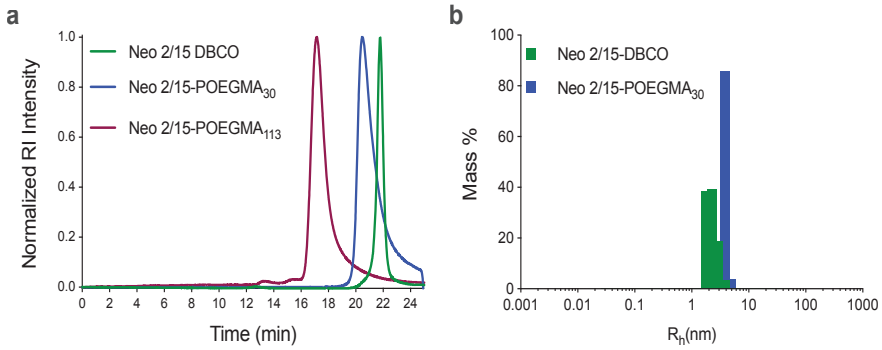



**Figure 2. Neo 2/15 and its site-specific and stoichiometric POEGMA conjugates are well-defined. (A)** SEC-MALS and **(B)** DLS analysis of Neo-2/15 conjugates. Indices show overall molecular weight of conjugates.

Having synthesized and characterized well-defined Neo 2/15-POEGMA and Neo 2/15-PEG conjugates, we next characterized their binding affinity to IL-2R components and compared them with IL-2 and IL-15. Neo 2/15-POEGMA$_{30}$ and Neo 2/15-POEGMA$_{113}$ conjugates have shown high binding affinity to human IL-2/15Rβ***γ*** with an equilibrium dissociation constant ($K_d$) of 45 and 613 pM, respectively, but a slightly lower $K_d$ than Neo 2/15 (37 pM) due to the steric hindrance imposed by the conjugated POEGMA, determined by SPR **(Table 1)**. We believe that the slightly lowered binding affinity will be an acceptable trade-off given the PK improvements will be made to the drug upon POEGMA conjugation. These results are more than competitive with human IL-2 and IL-15 that has a $K_d$ of 98 pM and 49 nM, respectively. Neo 2/15 and Neo 2/15-POEGMA conjugates have shown no affinity to IL-2Rα (data not shown).

**Table 1. Physical characterization of site-specific and stoichiometric Neo 2/15-POEGMA conjugates and their binding affinity to human IL2/15Rβ*γ*.** ($M_w$) molecular weight; ($Đ$) polydispersity index; ($R_h$) hydrodynamic size; ($K_d$) dissociation constant. *Calculated from the amino acid sequence. †Default value due to the monodisperse nature of the proteins.

| Compound | $M_w$ (kDa) | $Đ$ | $R_h$ (nm) | $K_d$ |
|---|---|---|---|---|
| Human IL-2 | 15.4* | 1.00† | 2.1 ± 0.3 | 98 pM |
| Human IL-15 | 12.8* | 1.00† | 1.8 ± 0.7 | 49 nM |
| Neo 2/15 | 12.9* | 1.00† | 2.2 ± 0.5 | 37 pM |
| Neo 2/15-POEGMA$_{30}$ | 29.6 | 1.04 | 3.3 ± 0.9 | 45 pM |
| Neo 2/15-POEGMA$_{113}$ | 113.1 | 1.22 | 5.6 ± 1.2 | 613 pM |

Finally, we tested the activity of the Neo 2/15 conjugates and compared them to murine IL-2 (+ controls) and PBS (- control) by measuring proliferation of murine CTLL-2 T-cells in an *in vitro* cell-based assay. The effective half-maximal dose ($EC_{50}$) of each Neo 2/15 variant was determined by fitting the dose-response curve to a four-parameter logistic, nonlinear regression model using GraphPad Prism 9 software. The Neo-POEGMA conjugates showed high potency in proliferating CTLL-2 cells ($EC_{50}$= 242 nM at 30 kDa and 89.4 nM at 65 kDa), but slightly lower potency than IL-2 ($EC_{50}$=0.27 nM) and IL-15 ($EC_{50}$=0.58 nM) (data not shown).

***PD-L1BP and its elastin-like polypeptide (ELP) fusions selectively bind PD-L1 with high affinity and show high pharmacological activity.*** Therapeutic binding proteins may better penetrate into solid tumors than mAbs, owing to their much smaller size and flexible structure.[18-19] In preliminary experiments, we have developed a PD-L1 binding fibronectin domain type III (Fn3) protein with 16.2 nM affinity for PD-L1 by selection from a diverse Fn3 phage display library that was created in-house followed by affinity maturation. Its avidity was further increased by creating linear oligomers of the Fn3 with valency ranging from 1–4. The PD-L1 binding affinity increased with increasing valency. The Fn3 tetramer, subsequently referred to as PD-L1BP, has a remarkably high binding affinity, with a $K_d$ of 36 pM and 25 pM to human and murine PD-L1, respectively, determined using SPR (**Figure 3a**). PD-L1BP showed no off-target binding to PD-L2 or PD-1 (data not shown).

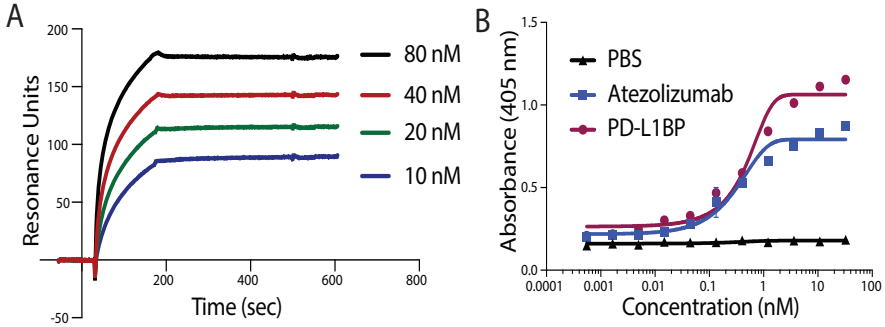



**Figure 3. PD-L1BP has high binding affinity to PD-L1 and high pharmacological activity. (A)** Binding affinity of PD-L1BP to human PD-L1 determined using SPR. **(B)** Pharmacological activity of PD-L1BP and atezolizumab determined by a cell-based assay.

Pharmacological activity of PD-L1BP was tested in a cell-based PD-L1 neutralization reporter assay. Briefly, the assay consists of two genetically engineered cell lines, Jurkat/PD-1 reporter cell and CHO TCR/PD-L1 cells. When co-cultured, the PD-1/PD-L1 interaction inhibits TCR-mediated luminescence via activation of the NFAT pathway. When the PD-1/PD-L1 interaction is disrupted by an FN3 or an anti-PD-L1 antibody, TCR activation induces luminescence that can be readily detected. We found that PD-L1BP shows strong inhibition of PD-1/PD-L1 activity in a cell assay with a half-maximal inhibitory concentration ($IC_{50}$) of 240 pM **(Figure 3b)**. These results are more than competitive with an FDA approved mAb that bind PD-L1 —atezolizumab— that has a $K_d$ of 433 pM for human PD-L1[20-21] and 134 pM for murine PD-L1[20] and an $IC_{50}$ of 140 pM **(Figure 3b).**

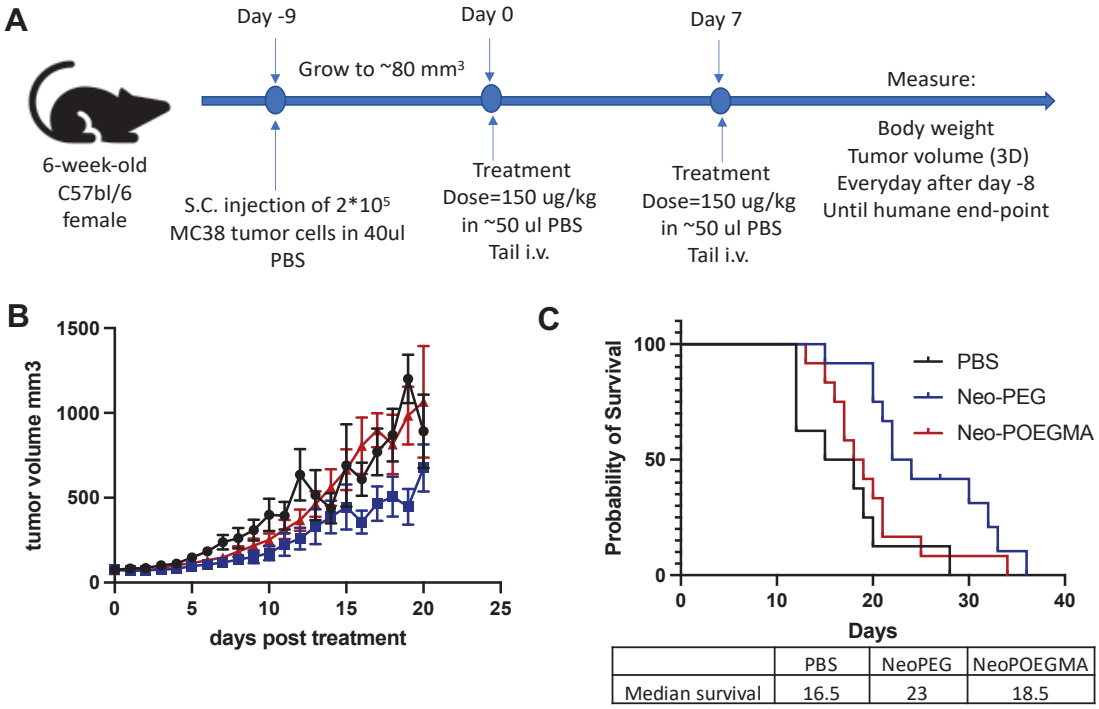
Motivated by these results, we next aimed to synthesize POEGMA conjugates of PD-L1BP. It was cloned and expressed in *E. coli* with a C-terminus sortase-A recognition site (LPETG) and a polyhistidine tag, yielding PD-L1BP-LPETG-$His_6$. Although the protein was expressed and purified with high yield and purity, sortase-A enzyme did not process PD-L1BP to install a biorthogonal end group at its C-terminus. Given that the sortase-mediated bio-orthogonal handle attachment was successfully completed for Neo 2/15-LPETG-$His_6$ but not for PD-L1BP-LPETG-$His_6$, we attributed these results to larger $M_w$ of PD-L1BP-LPETG-$His_6$ (~43 vs. ~13 kDa). We next cloned PD-L1BP with a flexible linker, yielding PD-L1BP-GSAGSAAGSGEF-LPETG-$His_6$, to move the sortase recognition site away from PD-L1BP. Unfortunately, sortase-A did not recognize the resulting protein, indicating that sortase-mediated biorthogonal handle installment was not a viable conjugation methodology for attaching POEGMA to PD-L1BP.

**Preliminary Data on Aim 2**

***Neo 2/15 conjugates have a minimal anticancer efficacy in syngeneic MC38 colon cancer model.*** The optimal dose and efficacy of a PEGylated Neo 2/5 were shown by another research group prior to the start of this research project.[14] This development necessitated us to make slight changes on the experimental design. Specifically, it eliminated the need for the PK (Subtask 1.7) and dose (Subtask 2.5) optimization experiments but necessitated the benchmarking of Neo 2/15-POEGMA to the reported PEGylated Neo 2/15.

Having synthesized and characterized the preliminary Neo 2/15-POEGMA and Neo 2/15-PEG conjugates, we next synthesized Neo 2/15-PEG and Neo 2/15-POEGMA at large scale for animal studies using 40 kDa PEG and 52 kDa POEGMA, respectively. This PEG $M_w$ was chosen to match the PEGylated Neo 2/15 reported in the literature.[21] POEGMA $M_w$ was chosen based on previous studies done by our group where 52kDa was found to be the optimal.[18] The resulting conjugates were characterized, purified to remove endotoxin using high-capacity endotoxin removal columns (Pierce), lyophilized, and stored at -80ºC until animal studies.

The resulting conjugates were next tested in tumor-bearing mice to show if they differed in terms of efficacy. Briefly, MC38 cells were cultured and authenticated, followed by subcutaneously (*s.c.*) administrated into C57BL/6J mice ($2*10^5$ cells in 90 µl phosphate buffered saline (PBS)). When tumor volume reached ~80 $mm^3$, Neo 2/15-POEGMA and Neo 2/15-PEG were administrated intravenously (*i.v.)* into the tumor-bearing mice ($n$=12) every 7 days, twice (150 µg/kg, 50 µl PBS) using an equivalent injection volume of PBS as a negative control. Tumor volume and body weight were measured daily, and the data recorded to date was given in **Figure 4**.




| | PBS | NeoPEG | NeoPOEGMA |
|---|---|---|---|
| Median survival | 16.5 | 23 | 18.5 |

**Figure 4. Benchmarking Neo 2/15-POEGMA to the PEGylated Neo 2/15. (A)** In vivo study experimental scheme, and **(B)** tumor volume and **(C)** survival data.

Interestingly, the data showed no statistical difference among the treatments in terms of tumor volume and survival, with Neo-PEG showing a trend towards smaller tumor volume and longer survival. These preliminary data suggested possible degradation of the Neo 2/15 conjugates before the start of the experiment, given that the Neo 2/15-PEG conjugate had been reported to show significant ($p<0.0001$) anti-cancer efficacy by day 21 in MC38 colon cancer model when PBS used as a control.[21]

**What opportunities for training and professional development has the project provided?**

**Acquired skills, competence, and experience:** Ms. Ozer has been mentored by Dr. Chilkoti, Dr. Nair and Dr. Antonia on all aspects of the study, such as bioconjugate development, building tumor models, animal study design, and data interpretation. Imran has been trained by Chilkoti and Nair lab members on all necessary experimental skills and assisted by Xinghai Li, who is experienced in animal experiments, to ensure high quality. With the mentorship and training provided, Ms. Ozer has accomplished her research goals laid out in the award application. In addition to Ms. Ozer, Ms. Sirohi has also received an extensive training. She has been mentored and trained by Ms. Ozer and Ms. Min on every aspect of the study, more specifically on polymer synthesis, molecular cloning, conjugate synthesis and characterization, and animal handling.

**Professional development:** Ms. Ozer and Ms. Sirohi have presented the latest results regularly in the weekly Chilkoti and Nair Lab meetings in addition to monthly Immunology subgroup meetings, which are open to the Chilkoti Lab members that focus on development of cancer immunotherapies. These presentations allowed them to develop collaborative and team building skills and hone their oral presentation and critical thinking skills. These opportunities have been an effective means of honing their abilities to design, conduct, and evaluate scientific work, thus preparing them for independence.

**How were the results disseminated to communities of interest?**

The results were disseminated to the Chilkoti and Nair Lab members regularly in the weekly lab meetings in addition to monthly Immunology subgroup meetings, which are open to the Chilkoti Lab members that focus on development of cancer immunotherapies and vaccines.

**What do you plan to do during the next reporting period to accomplish the goals?**

Nothing to report

## *4.* IMPACT:

**What was the impact on the development of the principal discipline(s) of the project?**

The **impact** of this research is **two**-fold. **First,** it solves the increasing prevalence of PEG immunogenicity and antigenicity that is casting a pall on the development of PEGylated therapeutics. The hyperbranched structure of POEGMA does not induce a POEGMA-specific immune response, presumably because its short OEG side chains do not crosslink B-cell receptors as PEG does. In addition to its lack of immunogenicity, POEGMA does not react with PEG antibodies, suggesting that it can be safely used in the pre-existing PEG antibody positive population, which is now rampant because of chronic exposure to PEG through excipients in drugs and consumer products. These findings are highly topical because up to 70% of the human population has pre-existing antibodies to PEG,[22] and billions of individuals are in the process of being immunized with a PEG-containing liposome formulation of the Pfizer and Moderna vaccines for COVID-19. There is also a growing consensus that PEG is responsible for the immediate-type hypersensitivity reactions[23,24] seen with the Moderna and Pfizer COVID-19[33] and other vaccines.[34] **Second,** we have showcased the utility of this platform in treating colorectal cancer while solving the toxicity, ambiguous clinical activity, and short blood circulation time problems of cytokines. This is of impact as colorectal cancer has high incidence and mortality among Service members and Veterans[35] as well as millions of Americans.[36]

**What was the impact on other disciplines?**

We believe that these results may herald the emergence of a new era of synthetic materials that leverages the power of polymer chemistry and smart material design. These polymers are likely to be useful for a range of applications that can benefit from non-immunogenic materials. Because these materials are chemically conjugated to biologics, they can be used to formulate a myriad of biologics to endow them with favorable blood circulation and efficacy.

**What was the impact on technology transfer?**

The next-generation POEGMA conjugate technology had already been patented and translated out of Duke University as a startup company founded by Dr. Ashutosh Chilkoti. These results have further solidified the utility of this technology on creating POEGMA conjugates of biologics and formed the first example of cytokine-POEGMA conjugates.

**What was the impact on society beyond science and technology?**

Nothing to report.

## 5. CHANGES/PROBLEMS:

There were **two** changes to this research. **First,** the optimal dose and efficacy of a PEGylated Neo 2/5 were shown by another research group prior to the start of this research project. This development necessitated us to make slight changes on the experimental design. Specifically, it eliminated the need for the PK (Subtask 1.7) and dose (Subtask 2.5) optimization experiments but necessitated the benchmarking of Neo 2/15-POEGMA to the PEGylated Neo 2/15. **Second,** sortase enzyme could not process PD-L1BP to install a bio-orthogonal end group for site-specific and stoichiometric POEGMA conjugation. This finding led us to use elastin-like polypeptide (ELP) technology to impart long-acting behavior to PD-L1BP. In preliminary experiments, we showed that PD-L1BP-ELP fusion had a pharmacological activity and efficacy similar to that of Atezolizumab (data not shown), indicating the viability of ELP technology.

**Actual or anticipated problems or delays and actions or plans to resolve them**

The problem related to the POEGMA installation on PD-L1BP was resolved by using ELP technology. Briefly, ELP is genetically encoded and readily expressed as a C- terminal fusion of PD-L1BP in *E. coli*, yielding monodisperse PD-L1BP-ELP fusions. ELPs are useful as a drug delivery platform as they increase overall size of their fusion partners above renal kidney excretion threshold and prolong blood circulation half-life. Chilkoti Lab has previously shown utility of ELP drug delivery technology in a preclinical setting by developing an ELP fusion of glucagon-like peptide-1 (GLP1), a peptide drug with very short (~2 min) half-life used in treating type-2 diabetes, which provided 10 days of blood glucose control in diabetic mice.[26] This level of blood glucose control was more than competitive with that provided by two FDA approved sustained release technologies—Trulicity, an Fc fusion of GLP-1, and Bydureon, a degradable microsphere formulation, showing that ELP fusions are long-acting and can perform as well as an antibody-based drug. ELP conjugates have also been injected into humans in several clinical trials (Phase 2b; NCT04122170 and NCT03556020) and have shown high efficacy.

**Changes that had a significant impact on expenditures**

The removal of the PD-L1BP-POEGMA conjugate synthesis from the experimental design has enabled us to spend less than anticipated on research animals and husbandry.

**Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

**Significant changes in use or care of human subjects**

Nothing to report
IRB approval date: 06/25/2020
HRPO approval date: 09/10/2020

**Significant changes in use or care of vertebrate animals**

Nothing to report
IACUC approval date: 04/29/2020
ACURO approval date: 06/26/2020 (renewed on 01/28/2021)

**Significant changes in use of biohazards and/or select agents**

| |
|---|
| Nothing to report |

## 6. PRODUCTS:

- **Publications, conference papers, and presentations**

  **Journal publications.**

| |
|---|
| Nothing to report |

**Books or other non-periodical, one-time publications.**

| |
|---|
| Nothing to report |

**Other publications, conference papers and presentations**.

| |
|---|
| Nothing to report |

- **Website(s) or other Internet site(s)**

| |
|---|
| Nothing to report |

- **Technologies or techniques**

| |
|---|
| We have showed the utility of the next-generation POEGMA conjugate technology on synthesizing cytokine-POEGMA conjugates for the first time in this research project. These results solidify the power of POEGMA conjugate technology to replace immunogenic PEGylation. |

- **Inventions, patent applications, and/or licenses**

| |
|---|
| Nothing to report |

- **Other Products**

| |
|---|
| The data detailed earlier is the major reportable outcome of this research project. We are in the process of compiling the data and share out findings with the scientific community. |

## 7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

**What individuals have worked on the project?**

| | |
|---|---|
| *Name:* | *Imran Ozer* |
| *Project Role:* | *Principal Investigator* |
| *Nearest person month worked:* | *12* |
| *Contribution to Project:* | *Ms. Ozer has performed work in all areas of the project. She has had an integral role in polymer and conjugate synthesis and characterization as well as the design of the animal studies.* |
| *Name:* | *Ashutosh Chilkoti* |
| *Project Role:* | *Mentor* |
| *Nearest person month worked:* | *1* |
| *Contribution to Project:* | *Dr. Chilkoti mentored the PI on coordinating the project and played a key role in experimental design and interpretation of the data.* |
| *Funding Support:* | *Duke University* |
| *Name:* | *Smita Nair* |
| *Project Role:* | *Co-mentor* |
| *Nearest person month worked:* | *1* |
| *Contribution to Project:* | *Dr. Nair played a key role in vivo study design.* |
| *Funding Support:* | *Duke University* |
| *Name:* | *Scott J. Antonia* |
| *Project Role:* | *Co-mentor* |
| *Nearest person month worked:* | *1* |
| *Contribution to Project:* | *Dr. Antonia played a key role in vivo study design.* |
| *Funding Support:* | *Duke University* |
| *Name:* | *Parul Sirohi* |
| *Project Role:* | *Graduate Student* |
| *Nearest person month worked:* | *3* |
| *Contribution to Project:* | *Ms. Sirohi has performed mid-scale synthesis and endotoxin purification of drug-POEGMA conjugate, and assisted the PI during the in vivo experiments.* |
| *Funding Support:* | *Duke University* |
| *Name:* | *Junseon Min* |
| *Project Role:* | *Graduate Student* |
| *Nearest person month worked:* | *3* |
| *Contribution to Project:* | *Ms. Min has performed binding affinity determination using Surface Plasmon Resonance.* |
| *Funding Support:* | *Duke University* |

**Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Nothing to report

**What other organizations were involved as partners?**

Nothing to report

## 8. SPECIAL REPORTING REQUIREMENTS

**COLLABORATIVE AWARDS:**

**QUAD CHARTS:**

## 9. APPENDICES:

**References**


1 Ganesh, K. *et al.* Immunotherapy in colorectal cancer: rationale, challenges and potential. *Nature Reviews Gastroenterology & Hepatology* **16**, 361-375, (2019).

2 Ott, P. A., Hodi, F. S., Kaufman, H. L., Wigginton, J. M. & Wolchok, J. D. Combination immunotherapy: a road map. *Journal for ImmunoTherapy of Cancer* **5**, 16, (2017).

3 Deng, R. *et al.* Preclinical pharmacokinetics, pharmacodynamics, tissue distribution, and tumor penetration of anti-PD-L1 monoclonal antibody, an immune checkpoint inhibitor. *MAbs* **8**, 593-603, (2016).

4 Gorman, K., McGinnis, J. & Kay, B. Generating FN3-Based Affinity Reagents Through Phage Display. *Current Protocols in Chemical Biology* **10**, e39, (2018).

5 Levisson, M., Spruijt, R. B., Winkel, I. N., Kengen, S. W. M. & van der Oost, J. in *Protein Downstream Processing: Design, Development and Application of High and Low-Resolution Methods* (ed Nikolaos E. Labrou) 211-229 (Humana Press, 2014).

6 Zhang, M. *et al.* IL-15 enhanced antibody-dependent cellular cytotoxicity mediated by NK cells and macrophages. *Proceedings of the National Academy of Sciences* **115**, E10915, (2018).

7 O'Sullivan Coyne, G. H. *et al.* Phase I study of recombinant interleukin-15 in combination with checkpoint inhibitors nivolumab and ipilimumab in subjects with refractory cancers. *Journal of Clinical Oncology* **36**, TPS3128-TPS3128, (2018).

8 Robinson, T. O. & Schluns, K. S. The potential and promise of IL-15 in immuno-oncogenic therapies. *Immunology letters* **190**, 159-168, (2017).

9 Jiang, T., Zhou, C. & Ren, S. Role of IL-2 in cancer immunotherapy. *Oncoimmunology* **5**, e1163462-e1163462, (2016).

10 Rosenberg, S. A. *et al.* Observations on the Systemic Administration of Autologous Lymphokine-Activated Killer Cells and Recombinant Interleukin-2 to Patients with Metastatic Cancer. *New England Journal of Medicine* **313**, 1485-1492, (1985).

11 Rosenberg, S. A. IL-2: The First Effective Immunotherapy for Human Cancer. *The Journal of Immunology* **192**, 5451, (2014).

12 Maker, A. V. *et al.* Tumor Regression and Autoimmunity in Patients Treated With Cytotoxic T Lymphocyte–Associated Antigen 4 Blockade and Interleukin 2: A Phase I/II Study. *Annals of Surgical Oncology* **12**, 1005-1016, (2005).

13 Hannani, D. *et al.* Anticancer immunotherapy by CTLA-4 blockade: obligatory contribution of IL-2 receptors and negative prognostic impact of soluble CD25. *Cell Research* **25**, 208, (2015).

14 Carl Walkey, P. R. S., BS; Umut Ulge, MD, PhD; Daniel Adriano Silva Manzano, PhD; Jonathan G. Drachman, MD;. in *SITC 2020.* (ed Neoleukin Therapeutics).

15 Yao, X. *et al.* Levels of peripheral CD4(+)FoxP3(+) regulatory T cells are negatively associated with clinical response to adoptive immunotherapy of human cancer. *Blood* **119**, 5688, (2012).

16 Levin, A. M. *et al.* Exploiting a natural conformational switch to engineer an interleukin-2 'superkine'. *Nature* **484**, 529, (2012).

17 Ozer, I. & Chilkoti, A. Site-Specific and Stoichiometric Stealth Polymer Conjugates of Therapeutic Peptides and Proteins. *Bioconjugate Chemistry*, (2016).

18 Qi, Y. *et al.* A brush-polymer/exendin-4 conjugate reduces blood glucose levels for up to five days and eliminates poly(ethylene glycol) antigenicity. *Nature Biomedical Engineering* **1**, 0002, (2016).

19 Pang, Y., Liu, J., Qi, Y., Li, X. & Chilkoti, A. A Modular Method for the High-Yield Synthesis of Site-Specific Protein-Polymer Therapeutics. *Angew Chem Int Ed Engl* **55**, 10296-10300, (2016).

20 J., B. J., Jayanta, B. & Ashutosh, C. A Noncanonical Function of Sortase Enables Site-Specific Conjugation of Small Molecules to Lysine Residues in Proteins. *Angewandte Chemie International Edition* **54**, 441-445, (2015).

21 Carl Walkey, L. T., Kevin Yu, Paul Hara, Ryan Swanson, Umut Ulge, Jonathan Drachman, and Daniel-Adriano Silva; Neoleukin Therapeutics, Seattle WA. in *AACR Annual Meeting II* (ed Neoleukin Therapeutics).

22 Yang, Q. *et al.* Analysis of Pre-existing IgG and IgM Antibodies against Polyethylene Glycol (PEG) in the General Population. *Analytical Chemistry* **88**, 11804-11812, (2016).

23 Wenande, E. & Garvey, L. H. Immediate-type hypersensitivity to polyethylene glycols: a review. *Clinical & Experimental Allergy* **46**, 907-922, (2016).

24 Sellaturay, P., Nasser, S. & Ewan, P. Polyethylene Glycol-Induced Systemic Allergic Reactions (Anaphylaxis). *J Allergy Clin Immunol Pract*, (2020).

25 Silva, D.-A. *et al.* De novo design of potent and selective mimics of IL-2 and IL-15. *Nature* **565**, 186-191, (2019).

26 Luginbuhl, K. M. *et al.* One-week glucose control via zero-order release kinetics from an injectable depot of glucagon-like peptide-1 fused to a thermosensitive biopolymer. *Nat Biomed Eng* **1**, (2017).

27 Hershfield, M. S. *et al.* Induced and pre-existing anti-polyethylene glycol antibody in a trial of every 3-week dosing of pegloticase for refractory gout, including in organ transplant recipients. *Arthritis Res Ther* **16**, R63, (2014).

28 Ganson, N. J., Kelly, S. J., Scarlett, E., Sundy, J. S. & Hershfield, M. S. Control of hyperuricemia in subjects with refractory gout, and induction of antibody against poly(ethylene glycol) (PEG), in a phase I trial of subcutaneous PEGylated urate oxidase. *Arthritis Res Ther* **8**, R12, (2006).

29 Ganson, N. J. *et al.* Pre-existing anti-polyethylene glycol antibody linked to first-exposure allergic reactions to pegnivacogin, a PEGylated RNA aptamer. *J Allergy Clin Immunol* **137**, 1610-1613 e1617, (2016).

30 Armstrong, J. K. *et al.* Antibody against poly(ethylene glycol) adversely affects PEG-asparaginase therapy in acute lymphoblastic leukemia patients. *Cancer* **110**, 103-111, (2007).

31 Garay, R. P., El-Gewely, R., Armstrong, J. K., Garratty, G. & Richette, P. Antibodies against polyethylene glycol in healthy subjects and in patients treated with PEG-conjugated agents. *Expert Opin Drug Deliv* **9**, 1319-1323, (2012).

32 Richter, A. W. & Akerblom, E. Polyethylene glycol reactive antibodies in man: titer distribution in allergic patients treated with monomethoxy polyethylene glycol modified allergens or placebo, and in healthy blood donors. *Int Arch Allergy Appl Immunol* **74**, 36-39, (1984).

33 Garvey, L. H. & Nasser, S. Anaphylaxis to the first COVID-19 vaccine: is polyethylene glycol (PEG) the culprit? *Br J Anaesth*, (2020).

34 Badiu, I., Geuna, M., Heffler, E. & Rolla, G. Hypersensitivity reaction to human papillomavirus vaccine due to polysorbate 80. *BMJ Case Rep* **2012**, (2012).

35 Zullig, L. L. *et al.* Cancer Incidence Among Patients of the U.S. Veterans Affairs Health Care System: 2010 Update. *Military Medicine* **182**, e1883-e1891, (2017).

36 Colorectal Cancer Facts & Figures 2017-2019. (Atlanta, 2017).