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# Tannic Acid-Containing Nanothin Coatings Dampen Innate Immune-Derived Pro-Inflammatory Chemokine Synthesis

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## Abstract

Type 1 Diabetes (T1D) is a chronic pro-inflammatory autoimmune disease consisting of reactive oxygen species (ROS), pro-inflammatory cytokines, and islet-infiltrating leukocytes involved in pancreatic β-cell lysis. One promising treatment for T1D is islet transplantation; however, its clinical application is constrained due to limited islet availability, adverse effects of immunosuppressants on islet function, and declining graft survival. Islet encapsulation may provide an immunoprotective barrier to help preserve islet function and prevent immune-mediated rejection after transplantation into T1D patients. We previously demonstrated that a novel cytoprotective nanothin coating for islet encapsulation consisting of tannic acid (TA), an immunomodulatory antioxidant, and poly N-vinylpyrrolidone (PVPON), was efficacious in dampening diabetogenic CD4 T cell and macrophage responses involved in transplant rejection. Therefore, we hypothesized that in addition to suppressing pro-inflammatory cytokine synthesis, TA/PVPON would similarly blunt the production of pro-inflammatory chemokines involved in recruiting immune cells to the site of islet engraftment. Our results provide further support that TA/PVPON-containing encapsulated islets are effective in

suppressing pro-inflammatory CCL5 and CXCL10 chemokine synthesis. The use of novel TA/PVPON nanothin coatings may potentially decrease immune-mediated responses and enhance islet allo- and xenograft acceptance to restore euglycemia in T1D patients.

Key words: Type 1 Diabetes, chemokines, tannic acid, immunosuppressant, islet transplantation

**Introduction**

Type 1 Diabetes (T1D) is a T cell-mediated autoimmune disease characterized by the targeted lysis of insulinproducing pancreatic β-cells. Patients with T1D are genetically predisposed to autoimmunity, but mounting evidence suggests an environmental trigger is required to instigate the development of autoimmunity[1]. As a result of insulin loss, T1D patients are unable to adequately maintain normal blood glucose levels; thus, patients regulate blood glucose levels via daily insulin injections or an insulin pump. Numerous complications arise as a result of insulin deficiency, including cardiovascular disease, nephropathy, and retinopathy. Another viable alternative to insulin injection is pancreatic islet transplantation, a process that involves the isolation of islets from cadaveric donors and the transplantation of those islets into T1D patients[2]. One of the main advantages of islet transplantation is that the pancreatic β-cell is finely tuned to properly regulate glucose levels within the body, and episodes of hyperglycemia (elevated blood glucose) and hypoglycemia (low blood glucose) are less frequent. Wide fluctuations in blood glucose can significantly alter metabolism and contribute to life-threatening diabetic complications.

The landmark Edmonton protocol demonstrated great promise that islet isolation from multiple cadaveric donors and transplantation into the portal vein with a glucocorticoidfree immunosuppressive cocktail could restore euglycemia in Type 1 diabetic patients without the use of exogenous insulin therapy[2]. Transplant recipients require immunosuppressants that include injections of daclizumab for a period of 8 weeks and sirolimus (daily) to protect the donor's islets from being rejected[2]. Unfortunately, immunosuppressants can weaken the immune system and compromise islet function; therefore, attempts to transplant islets without immunosuppressive therapies and/or develop novel immunotherapies with low toxicity are highly desired.

Prior studies have utilized various methods of islet encapsulation for immunoprotection including macroencapsulation with alginate or polyethylene glycol (PEG), polymembrane pouches within an oxygenated chamber, and microencapsulation in high viscous alginate droplets[3]. However, there are significant drawbacks as macro- and microencapsulation with alginate requires extreme purity since microbial contaminants in alginate can inadvertently activate the immune system and cause cell death[4]. Also, PEG hydrogels for islet encapsulation are large, degradable, and present a barrier for rapid transport of oxygen, nutrients, and therapeutic factors[5]. Therefore, a flexible and immunosuppressive barrier is needed to perform the task of protecting living islets while maintaining their functionality within the body.

One effective method of islet encapsulation utilizes the Layer-by-Layer (LbL) protocol of coating islets with nano-thin layers of water soluble polymers in aqueous solutions[4]. The LbL method of applying a thin coating on cells allows them to respond to stimuli, contains a

conformal and adjustable coating, and can be efficiently conjugated with specific inhibitors, proteins, and antibodies to modulate specific biochemical pathways. We have utilized this LbL method with biomaterials of tannic acid (TA) and PVPON (poly N-vinylpyrrolidone). TA is an antioxidant that scavenges free radicals and inhibits free radical-induced oxidation[4]. PVPON is a hydrophilic, non-toxic polymer which can function as an efficient scaffold. These features of TA/PVPON are important in hindering the production of reactive oxygen species (ROS) that are responsible for signaling and activating other immune cells. Our previous results demonstrated that TA/ PVPON encapsulation of pancreatic islets was non-toxic and maintained islet function and stability up to 7 days *in vitro*[6]. More importantly, we were also able to demonstrate that TA/ PVPON exhibited an immunosuppressive effect on activated macrophages and autoreactive T cells by dampening the synthesis of innate immune-derived pro-inflammatory cytokines and adaptive immune T cell effector responses involved in islet rejection[4,7].

Chemokines play an integral role in the pathogenesis of Type 1 Diabetes and islet transplant rejection. They are a specific type of protein that drives cellular chemotaxis, which induces pro-inflammatory cell migration to a specific region in the body. This use of chemotaxis can directly drive immune cells to the site of newly transplanted islets and cause pancreatic β-cell necrosis. There are many chemokines associated with T1D, but one widely known chemokine is CXCL10, which has been identified as a dominant chemokine involved in murine models of T1D and human T1D[8]. RANTES, also known as CCL5, plays a key role in T cell proliferation and activation of T cells in inflammatory sites of T1D patients. Zhernakova *et al.*[9] demonstrated that single-nucleotide polymorphisms (SNPs) in RANTES result in lower RANTES production in T1D. Results show that T1D carriers of RANTES SNPs exhibited lower levels of RANTES

compared to non-carriers and suggest that CCL5 chemokine play a role in the parthenogenesis of T1D[9]. Other chemokines such as CCL2, CCL3, and CCL4 are presented at lower levels than CCL5 and CXL10. However, they also play a crucial role in recruiting immune cells through chemotaxis.

Despite the immunotherapeutic potential of TA/PVPON encapsulation material for islet transplants, little is known regarding the effects on chemokine production. In an attempt to identify how TA/PVPON may regulate chemokine expression in macrophages, the NOD (Non-Obese Diabetic) mouse model of spontaneous T1D was utilized to generate bone marrow-derived macrophages[6]. Classically-activated macrophages, termed M1, are pro-inflammatory and possess specialized abilities to kill intracellular microbes and destroy β-cells[10]. These macrophages become activated in response to microbial products such as lipopolysaccharide (LPS) or viral double-stranded RNA upon engagement with extracellular or intracellular specialized toll-like receptors (TLR). Upon activation, pro-inflammatory cytokines specific for M1 activation such as TNF-α will be secreted; ROS and macrophage activation markers CD80 and CD86 will also be expressed on the cell surface. In contrast, M2, or alternativelyactivated macrophages, are involved in wound healing, allergic responses, and immune suppression. They will synthesize a different cytokine and chemokine profile, produce Arginase-1, and increase expression of scavenger receptors such as CD206[10]. Our results provide further evidence that the antioxidant and immunosuppressive properties of TA/ PVPON can elicit a decrease in pro-inflammatory chemokine production, decrease pro-inflammatory M1 macrophages phenotypes, and potentially protect encapsulated islets from transplant rejection.

## Materials and Methods

*Mice*

NOD.ShiLtJ mice were bred and housed at the Research Support Building of the University of Alabama at Birmingham under pathogen-free conditions and observing IACUC-approved mouse protocols[11]. Male mice (age 7-9 weeks) were used in all experiments.

*Tannic Acid and poly (n-vinylpyrrolidone) capsules (TA/PVPON)*

TA/PVPON capsules were synthesized as previously described[4]. The three capsules provided were named according to their size: capsules 2-5, 4-5, and 4-5.5. Hollow hydrogen-bonded capsules were prepared by coating 4.0 ± 0.1 µm and 2.0 ± 0.1 µm silica particles in diameter with tannic acid and PVPON through a layer-by-layer method[4,7]. Number 5 and number 5.5 represents either tannic acid or PVPON on the outermost layer, respectively. Therefore, the numbers 2-5 represents a 2 µm silica particle with tannic acid on the outermost layer, while 4-5.5 represents a 4 µm silica particle with PVPON on the outermost layer.

*Differentiation and stimulation of bone marrow-derived macrophages (BM-MΦ)*

Bone marrow hematopoietic stem cells were isolated from femurs and tibias of NOD mice and differentiated into macrophages using L-929 conditioned macrophage media as previously described[12]. Stem cells were plated in 24-well plates (1 mL per well), petri dishes (15 mL per petri), and chamber slides (350 µL per chamber) at a concentration of $1.0 \times 10^6$ cells $mL^{-1}$. After 7 days, differentiated macrophages were treated with macrophage media depleted of L-929

conditioned media for 24 hours prior to stimulation. Cells were then stimulated with 25 μg $mL^{-1}$ of the TLR3 ligand, poly(I:C), (low-molecular-weight double-stranded RNA synthetic analog InvivoGen) and treated with $1.0 \times 10^7$ counts $mL^{-1}$ of tannic acid and poly (N-vinylpyrrolidone) capsules at various time intervals[11].

*ELISA and Quantitative RT-PCR*

Chemokine expression was measured in the supernatant of untreated, poly(I:C)-, and TA/PVPON-treated macrophages. CCL2, CCL3, CCL4, CCL5, CCL17, CXCL10 was detected with a DuoSet ELISA kit (R&D Systems) according to the manufacturer's instructions. ELISA plates were read on a Synergy 2 microplate reader (BioTek) using Gen5 software.

RNA was isolated from poly(I:C)-stimulated and TA/PVPONtreated BM-MΦ using TRIzol (Invitrogen) and cDNA prepared by SuperScript III (Invitrogen) according to the manufacturer's protocol. The generated cDNA was amplified on a Roche LightCycler 480 instrument by quantitative PCR using the following TaqMan gene expression assays (Applied Biosystems): *Emr1* (Mm00802529), *Ccl2* (Mm00441242), *Ccl3* (Mm00441259), *Ccl4* (Mm00443111), *Ccl5* (Mm01302428), *Ccl17* (Mm00516136), *Cxcl10* (Mm00445235). The relative gene expression levels were calculated with $2^{-\Delta\Delta Ct}$ method, and *Emr1* was used as a housekeeping control gene for normalization[11]. The unstimulated samples were used as calibrator controls and set as 1.

*Flow Cytometry*

Prior to staining, Fcγ receptors of bone marrow-derived macrophages were blocked with Fc block (BD Biosciences) for 10 minutes at 4 °C and then incubated with fluorochromeconjugated

antibodies specific for F4/80, CD80, CD86, TNF-α, and Arg-1 (BD Biosciences, eBiosciences) for 30 minutes at 4 °C. Cells were collected on the Attune NxT Flow Cytometry (ThermoFisher) and analyzed with FlowJo (10.0.8r1) software (Tree Star, Inc.).

*Statistical analysis*

Data were analyzed using GraphPad Prism Version 5.0 statistical software. Determination of the difference between mean values for each experimental group was assessed using the 2-tailed t-test, with $p < 0.05$ considered significant. All experiments were performed at least three separate times with data obtained in triplicate wells in each experiment.

**Results**

*Pro-inflammatory chemokine expression was dampened in the presence of TA/PVPON*

To determine the immunosuppressive role of TA/PVPON in regulating chemokine expression of bone marrow-derived macrophages, the levels of mRNA accumulation and protein expression were measured by quantitative RT-PCR (qRT-PCR) and ELISA, respectively. We determined that the synthesis of CCL5 and CXCL10 protein and mRNA chemokine expression were lowered when p(I:C)-stimulated samples were treated with TA/PVPON capsules. At 48-hour, *Ccl5* mRNA levels via qRT-PCR were significantly reduced 1.8-fold with capsules 2-5 and 1.4-fold with 4-5, but no difference was observed with capsule 4-5.5 (Figure 1A). Similarly, in Figure 1B, mRNA levels at 48-hour for *Cxcl10* displayed significant reduction of 2.6-, 3.6-, and 1.6-fold with 2-5, 4-5, and 4-5.5, respectively. To corroborate the decrease in chemokine mRNA levels, CCL5 and CXCL10 protein levels were suppressed consistently over a period of 96 hours

(*data not shown*) by TA/PVPON capsules. At the 72-hour time point, CCL5 was reduced with capsule 2-5 at 1.8-fold while capsules 4-5 and 4-5.5 decreased CCL5 levels by 2-fold (Figure 2A). CXCL10 protein levels followed similar results with a 1.5-, 1.6-, and 1.4-fold reduction with capsules 2-5, 4-5, and 4-5.5, respectively (Figure 2B). Analyses of CCL2, CCL3, CCL4, and CCL17 mRNA and chemokine levels did not differ when p(I:C)-stimulated macrophages were treated with 2-5, 4-5, and 4-5.5 capsules (*data not shown*).

**Figure 1.**

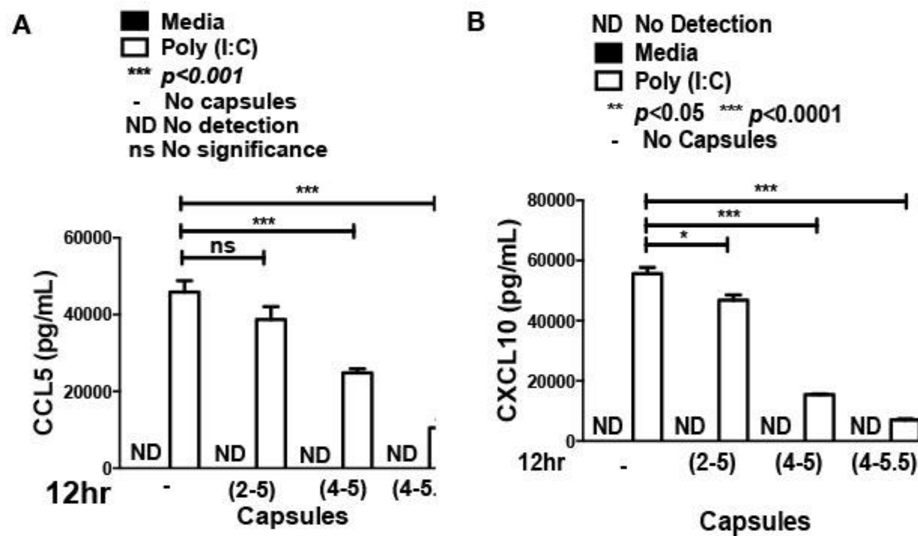


*Figure 1 | Ccl5 and Cxcl10 mRNA is reduced in poly(I:C)stimulated macrophages co-treated with TA/PVPON capsules. Ccl5 (A) and Cxcl10 (B) mRNA accumulation from TA/PVPON (at* $1.0 \times 10^7$ *cells* $mL^{-1}$*)-treated bone marrowderived macrophages was examined by qRT-PCR after 48-hour stimulation with 25 µg* $mL^{-1}$ *of poly(I:C). Results were normalized to no antigen/no capsules control group. Graphed data represent 3 independent experiments done in triplicates. With the standard deviation calculated as ns, not significant; ND, not detected.*

Figure 2.

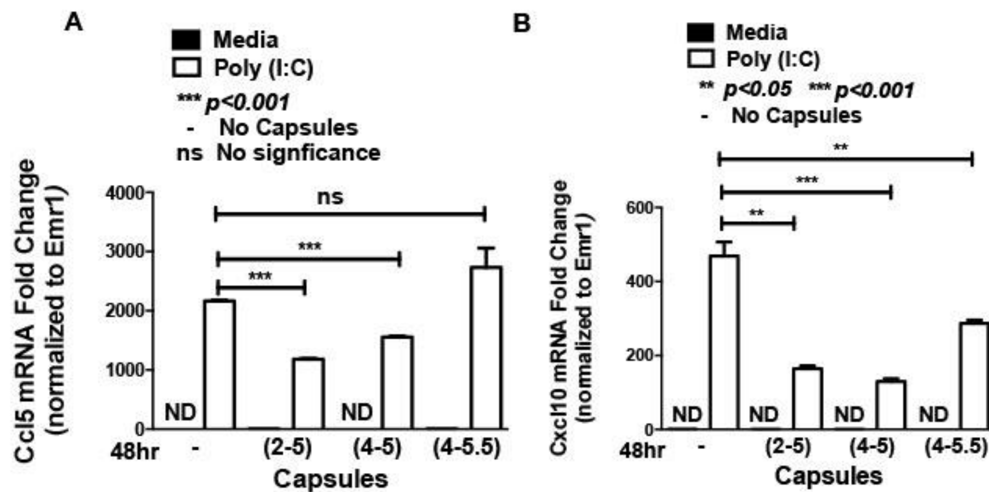


*Figure 2 | TA/PVPON capsules are efficient in decreasing CCL5 and CXCL10 chemokine expression. Supernatant of TA/PVPON (at 1.0 × $10^7$ cells $mL^{-1}$) treated bone marrowderived macrophages stimulated at 25 µg $mL^{-1}$ of poly(I:C) was examined via ELISA. CCL5 (A) and CXCL10 (B) chemokine expression at 72-hour were normalized to the no antigen/no capsules control group. Graphed data represent 3 independent experiments done in triplicates. Statistics was performed with a two-tailed Student's t-test and displayed as ns, not significant; ND, not detected.*

*M1 activation markers and pro-inflammatory cytokine were reduced while M2 activation marker was elevated in the presence of TA/PVPON*

To determine if bone marrow-derived macrophages exhibit reduced activation markers in the presence of TA/PVPON capsules, flow cytometric analysis of CD40, CD80, TNF-α, and Arg-1 expression levels with p(I:C)-stimulated macrophages was examined at the 24-hour time point. Shown in Figure 3 are gating strategies for the various macrophage markers. From the contour

FACS plots, the y-axis represents the expression of the macrophage specific marker, F4/80, and the x-axis displays M1 and M2 macrophage activation markers, CD80, CD86, TNF-α, and Arg-1. In the upper right hand quadrant for each plot, double positive cells expressing F4/80 and cell surface activation markers shown via percentage and geometric mean fluorescence intensity (gMFI) is shown. The levels of CD80, an extracellular M1 macrophage activation marker, exhibited a reduction after co-treatment with capsules 4-5 and 4-5.5 of 2.5- and 1.3-fold via gMFI, respectively (Figure 4). Similarly, with CD86, another M1 macrophage co-stimulatory molecule, the gMFI and percentage was reduced by 2.3- and 1.3-fold for capsules 4-5 and 4-5.5, respectively (Figure 5). In Figure 6 the level of TNF-α, an intracellular M1 pro-inflammatory cytokine, was also lowered in gMFI and percentage for all capsules by 1.4-fold (2-5), 3-fold (4-5), and 1.8-fold (4-5.5). In contrast, Arg-1, an M2 intracellular activation marker, was increased slightly via gMFI when treated with capsule 2-5, but showed no elevation with capsules 4-5 and 4-5.5 (Figure 7).

**Figure 3.**

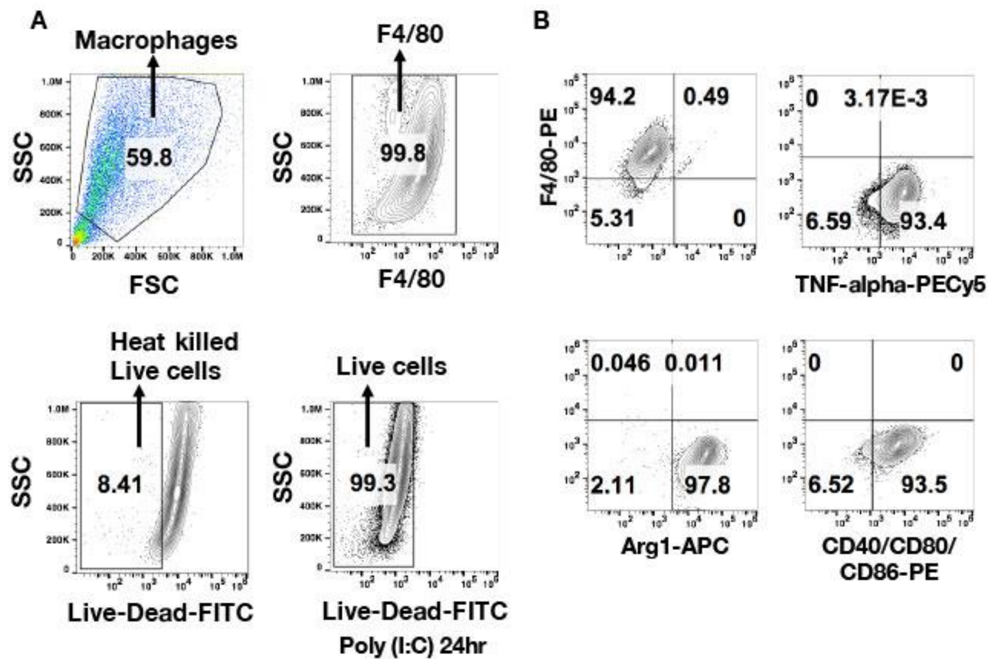


*Figure 3 | Gating strategies for F4/80 and macrophage activation markers via flow cytometry. A) Macrophages were gated using forward scatter side scatter profiles. Macrophages were heat-killed and gated via side scatter profile using a fixable live/dead APC stain (Invitrogen). F4/80 macrophage marker was gated via side scatter. Sample showing live cells gating was taken from poly(I:C) stimulated macrophages at 24-hour time point. B) Single color controls for macrophage markers. Each marker was stained with specific fluorochromes, PE, PeCy5, and APC, as labeled. F4/80 positive cells are located in the upper left-hand quadrant. The various markers are presented on the x-axis with bottom right quadrant representing positivity for particular marker.*

**Figure 4.**

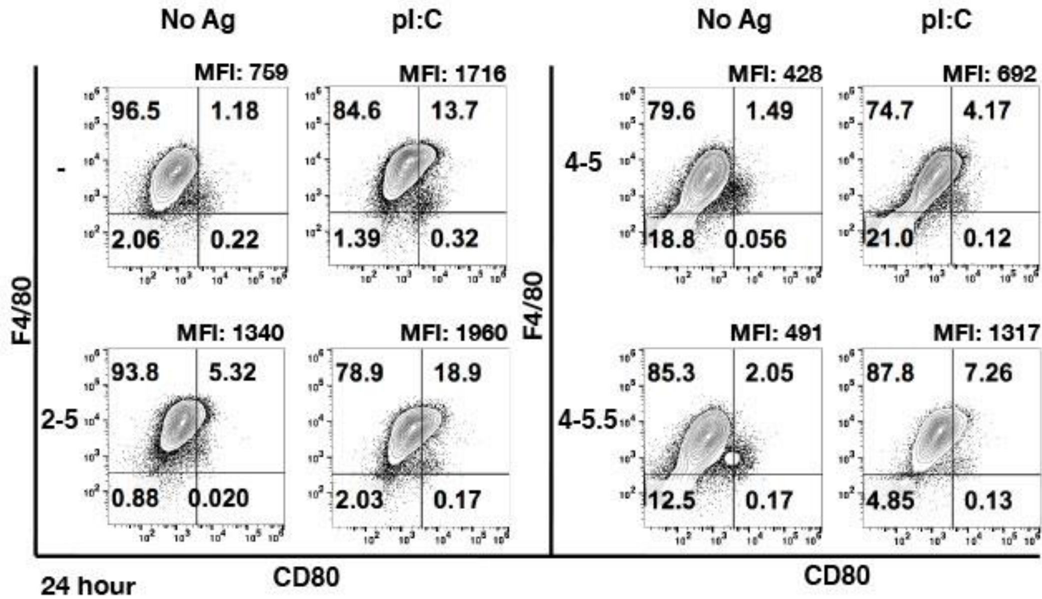


*Figure 4 | Macrophage activation marker, CD80, showed reduction in the presence of capsules. Flow cytometric analysis of CD80 by F4/80, a macrophage marker, is shown for 24 hours with TA/PVON (at 1.0 × $10^7$ cells $mL^{-1}$) treated bone marrow-derived macrophages stimulated with 25 μg $mL^{-1}$ of poly(I:C). CD80 was gated from live, F4/80 positive population of macrophages. FACS plots data represent 3 independent experiments done in triplicates.*

**Figure 5.**

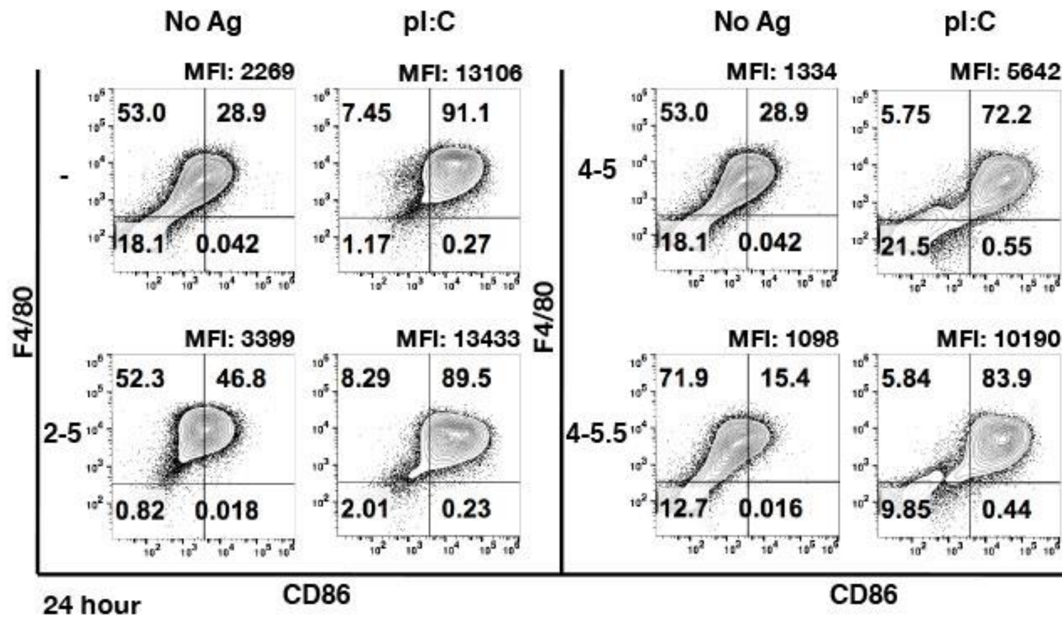


*Figure 5 | CD86, M1 macrophage activation marker, is suppressed in the presence of TA/PVPON. TA/PVON (at 1.0 × $10^7$ cells $mL^{-1}$) treated bone marrow-derived macrophages stimulated with 25 µg $mL^{-1}$ of poly(I:C) was examined using flow cytometry. Costimulatory marker CD86 was gated from the live, F4/80 positive population. Graphed data represent 3 independent experiments done in triplicates.*

**Figure 6.**

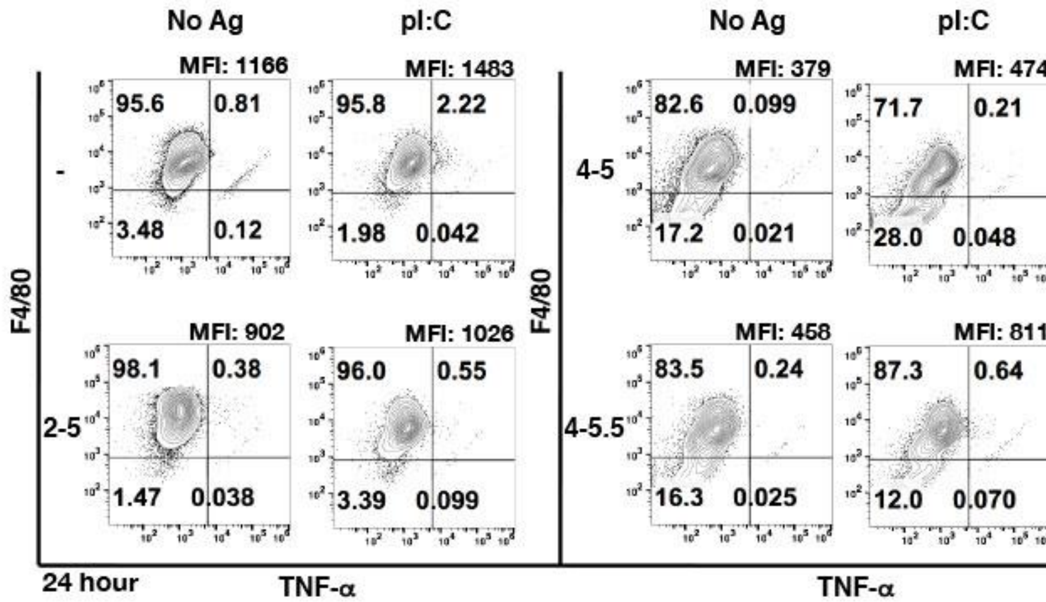


*Figure 6 | TA/PVPON capsules attenuate intracellular proinflammatory cytokine TNF-α expression. FACS plots were obtained at the 24-hour time point for TNF-α by F4/80 with 25 μg $mL^{-1}$ poly(I:C) stimulated bone marrow-derived macrophages introduced to capsules (at $1.0 \times 10^7$ cells $mL^{-1}$). TNF-α was gated from live, F4/80 positive population. Graphed data represent 3 independent experiments done in triplicates.*

**Figure 7.**

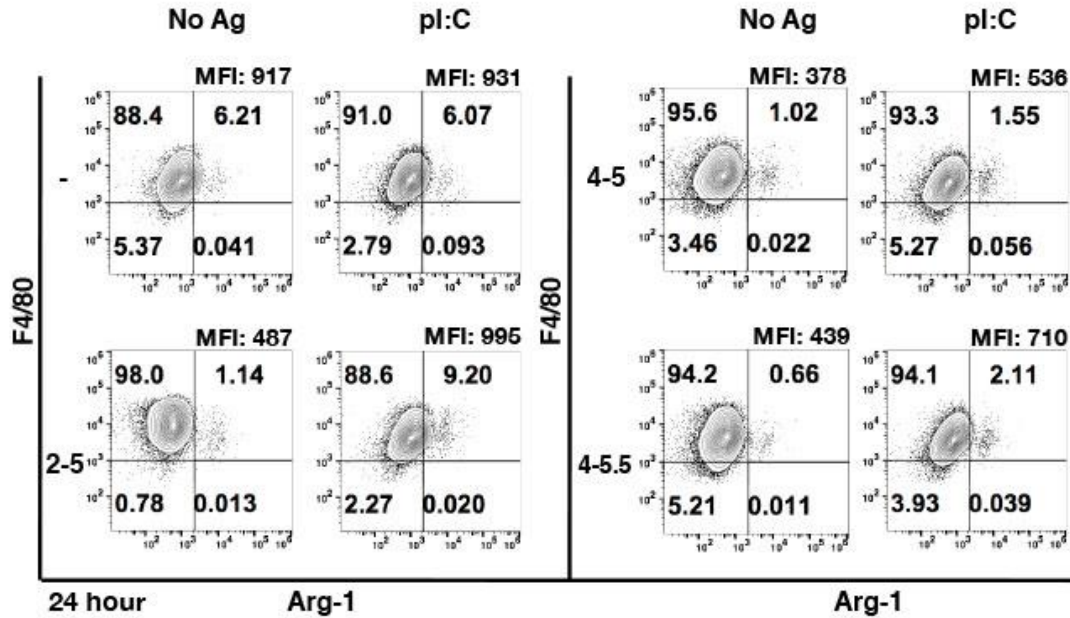


*Figure 7 | Arg-1 expression is elevated with the treatment of capsule 2-5. Flow cytometry analysis of Arg-1 by F4/80, macrophage marker, at 24-hour time point. TA/PVON (at $1.0 \times 10^7$ cells $mL^{-1}$) treated bone marrow-derived macrophages stimulated with 25 µg $mL^{-1}$ of poly(I:C) were examined. Arg-1 was gated from live, F4/80 positive population of macrophages. Graphed data represent 3 independent experiments done in triplicates.*

## Discussion

We have reported the importance of TA/PVPON capsules in dampening chemokine expression by ELISA and quantitative RT-PCR. With capsule treatments, both CXCL10 and CCL5 chemokines displayed decreased expression over a 96-hour time point. In addition, both chemokines presented significant decreases in mRNA accumulation at the transcriptional level. CXCL10 and CCL5 are important pro-inflammatory chemokines shown to correlate with T1D in

humans and in mice. In the serum of T1D patients, elevated CXCL10 levels were expressed suggesting that CXCL10 is a probable marker for predicting T1D[8]. For CCL5, elevated levels of CCL5 in mice splenocytes have shown to relate to the disease onset. As shown in Figure 1, the result displays capsule 2-5 exhibiting the most reduction in *Ccl5* and *Cxcl10* mRNA, followed by 4-5 and 4-5.5. From the data, we can conclude that thinner capsules containing tannic acid on their outer layer provide more immunosuppressive properties at the transcriptional level while thicker capsules provide more suppression at the translational level. In addition, it may be that thicker capsules, such as capsule 4-5.5, present their antioxidant properties much slower and more gradually than thinner capsules due to the PVPON being on the outermost layer. In contrast, at the protein level, Figure 2 displays capsule 4-5.5 exhibiting more reduction in CCL5 and CXCL10. From the results we can postulate that having PVPON on the outermost layer may delay the antioxidant properties of TA and provide a protective layer for the tannic acid layer to function properly over a longer span of time. Both layer size and composition of TA/PVPON capsules can contribute to the effectiveness of these capsules in suppressing the immune response. At both protein and mRNA levels, reduction in CXCL10 and CCL5 chemokines with the presence of TA/PVPON support the hypothesis that these capsules are immunosuppressive.

In addition to suppressing chemokine expression, macrophage activation markers CD80, CD86, and proinflammatory cytokine, TNF-α, were both dampened by TA/PVPON (Figures 4 and 5). Recall that CD80, CD86, and TNF-α are indicative of an M1 macrophage phenotype. Due to this fact, the expected result would be to observe the minimal CD80, CD86, and TNF-α expression. As predicted, decreased levels of CD86, CD80, and TNF-α suggest that TA/PVPON capsules 4-5 and 4-5.5 are reducing the level of M1 (pro-inflammatory macrophages) activation.

In addition, little to no reduction in CD80 and CD86 with capsule 2-5 may arise from the properties of the capsule. It may be that the thickness of capsule 2-5 is insufficient in dampening the activation markers presented by M1 macrophages. However, because CD80 and CD86 are extracellular M1 macrophage activation markers while TNF-α is an intracellular M1 activation marker, the reduction in these markers provide evidence that capsules 4-5 and 4-5.5 are effective in dampening M1 macrophage differentiation. With M2 macrophage activation, we would expect an increase in Arg-1 marker for all three capsules; however, only capsule 2-5 resulted in an increase at the 24-hour time point. These observations may suggest that with thinner capsules consisting of TA on the outermost layer, macrophages take on a more protective characteristic that is not seen in capsules 4-5 or 4-5.5. Overall, the decrease in CD80, CD86, and TNF-α does support the overarching hypothesis that thicker TA/PVPON capsules can dampen proinflammatory M1 macrophage differentiation and activation while supporting M2 macrophage differentiation with thinner capsules.

In our future studies, we want to test the *in vitro* and *in vivo* capability of TA/PVPON capsules through islet encapsulation and islet transplantation in NOD mice to determine if there is a decrease in chemokine expression as well as macrophage activation markers. We also want to observe pro-inflammatory chemokines such as CXCL10, CCL2, CCL3, CCL4, CCL5 and CCL17 that are involved in the inflammation process *in vivo*. We want to determine if there is any correlation in data between *in vitro* and *in vivo* experiments. With the findings from this experiment, further progress can be made in developing a novel material that can encapsulate and protect pancreatic β-cell islets from being targeted by the immune system. Furthermore, the

success of micro islet transplantation via stem cells or pancreatic cells can be sustained by the progress made in TA/PVPON research.

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