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Exploring Human Safe Compounds for Treatment in Type 1 Diabetes

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Introduction:

An estimated 11.3% percent or 37.3 million Americans suffer from either type 1 (T1D) or type 2 diabetes (T2D) differentiated based on the absolute or relative deficit of functional pancreatic β-cells, respectively. In addition to this staggering number, another 96 million Americans or 38.0% have type 2 related prediabetes making it one of the most common diseases the US [1][2]. Whereas T2D is associated with obesity and is characterized by impaired insulin regulated glucose uptake, both T1D and T2D have several notable complications if not treated properly such as cardiovascular disease, kidney disease, blindness, and even gum disease [3][4]. Although hypoglycemic complications are more pertinent to T1D and cardiovascular issues are more related to T2D, a lot of severe symptoms from diabetes are shared between the two types. For the past few decades, the number of affected individuals for all types of diabetes has been increasing, and yet the treatment for T1D disease has remained relatively unchanged [5]. Of the considerable array of prescribed diabetes drugs, insulin formulations for T1D, and for T2D, the biguanides, sulfonylureas, meglitinides, TZDs, DPP-4 inhibitors, GLP-1 mimetics, SGLT2 inhibitors [6], and their various combinations, many have shown long-term success but still host a wide range of potentially serious side effects.

Insulin and its various analogues still remain the most effective treatments for insulin dependent T1D and late stage T2D due to its essential role in glucose uptake within key metabolic tissues [7]. As a consequence of autoimmune mediated β-cell destruction through

inflammatory activation of CD4 and CD8 T-cells, there are persistent complications in treatment for T1D since the presence of autoantibodies to β-cell components persist regardless of insulin treatment [8]. With minimal clinical success of treating T1D with T2D specific alternatives, other approaches to circumvent the immune mediated destruction of β-cells are crucial in long-term management of the progressive disease.

The aforementioned anti-diabetes drugs are examples of treatments that target multiple tissues and treat disease after initial progression; however, there is growing emphasis on the roles of the nervous system, specifically the central nervous system, in glucose homeostasis, metabolic regulation, and maintenance of normal pancreatic endocrine function [9]. Research surrounding the agonistic action of nicotine in various tissues has demonstrated immune neutralizing effects [10] as well as indications of pro-survival/anti-inflammatory signaling. Previous experimental data demonstrating interruption of vagal innervation resulted in an 80% decrease in relative activity of Akt, a central mediator in β-cell growth pathways [11,12]. This suggests that parasympathetic innervation to β-cells plays a key role in sustaining proliferation as well as preventing reductions in β-cell mass, a counter response to immune-mediated destruction and a key index of accumulated functional β-cells [13]. Attempts at elucidating this role of neural signaling in pancreatic β-cells through the agonistic role of nicotine and synthetic analogs lead to studies demonstrating a reduction of the response of inflammatory cytokines and partial restoration of insulin release in a T1D model [14]. The innervation of the pancreas is a part of a system known as the autonomic nervous system (ANS), or more specifically the cholinergic system, an excitatory pathway involved in the parasympathetic stimulation of the pancreatic exocrine function and β-cell secretion [15]. Within this system there are two main types of acetylcholine receptors (AChR), muscarinic (mAChR) and nicotinic (nAChR) [16]. The nAChR's are composed

of 5 subunits of the following α1-10, β1-4, γ, δ, ε formed with radial symmetry and a central pore; each subunit includes four transmembrane α helixes, an intracellular domain, and an extracellular domain responsible for binding acetylcholine and other agonists [17]. One particular type of nAChR, the homopentamer α7-nAChR, is constitutively expressed in β-cells and agonist binding may lead to beneficial downstream effects (**Figure 1**).

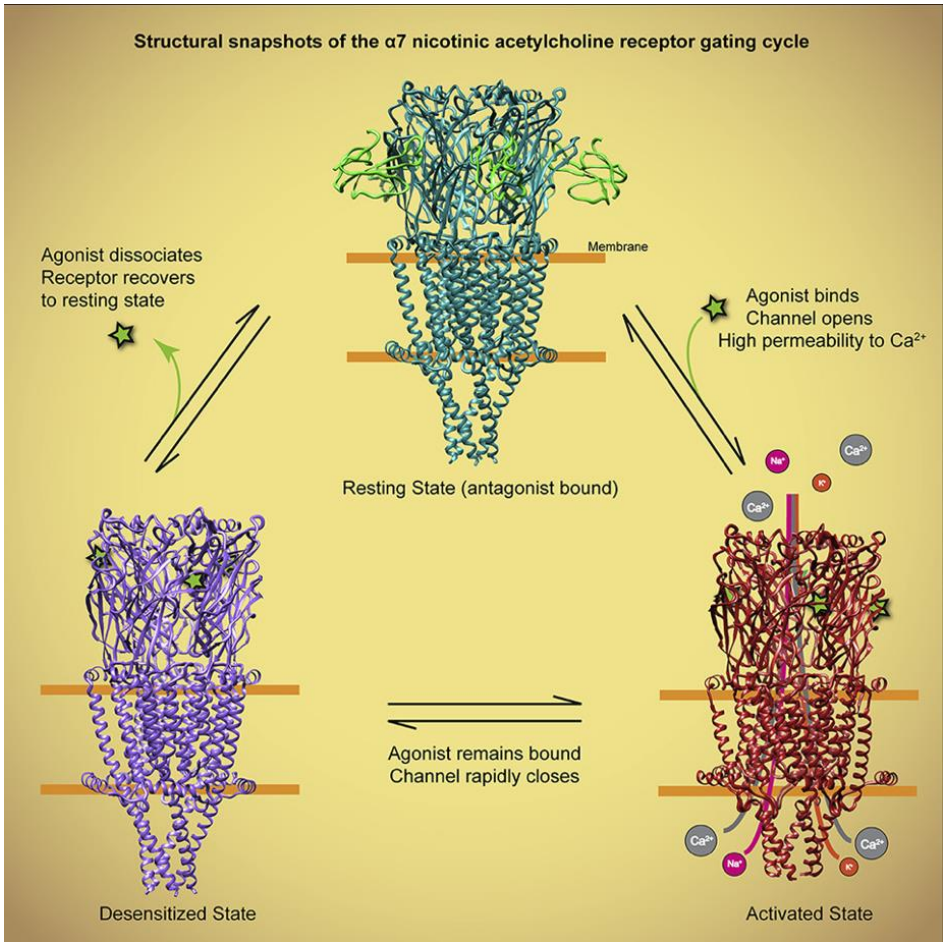

Figure 1 – Graphical abstract from Noviello and colleagues [17] outlining the structural composition and predicted function of the α7-nAChR in the presence of an agonist and in the presence of antagonist. Activated state demonstrates the ion channel's increased permeability to calcium ions which mediates its downstream effects.

Innervation of the pancreatic β-cell by the ANS is in part mediated via the α7-nAChR, and recent studies suggest it may be vital to the normal function and production of β-cells and, therefore, insulin [18]. In T1D, extensive research has gone into resolving the signaling cascades following immune-mediated destruction of pancreatic β-cells and the subsequent release of the pro-inflammatory cytokines (IL-1β, TNFα, and IFNγ) triggering NF-κβ–mediated stimulation of iNOS [19]. These cytokines at physiological concentrations have been shown to increase T1D progression [20] while at much higher concentrations, specifically shown with IFNγ, can paradoxically have a preventative role in T1D progression [21]. Therefore, it is important to demonstrate the effectiveness of these specific agonists with regard to pathophysiologically relevant concentrations of pro-inflammatory cytokines. Previous studies centered on acetylcholine manipulation and reduced inflammation showed success within areas innervated by the vagus nerve suggesting the β-cell α7-nAChR may serve as a target for pharmacological manipulation by reducing cytokine mediated inflammatory activity through reduction of iNOS (playing a key role in autoimmune destruction) [22] through the JAK2/STAT3 cascade, [23,24] and/or through increases in pro-survival signaling (through CREB/Akt mediated pathways) [15,25] (**Figure 2**).

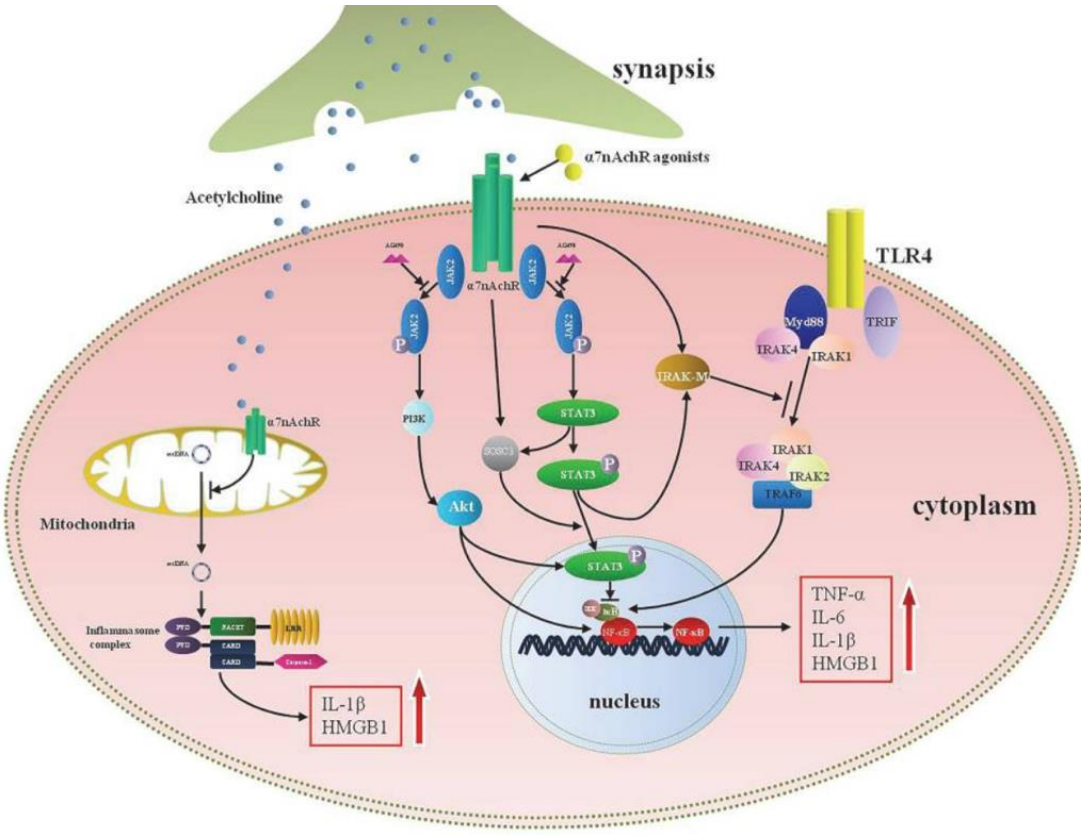

Figure 2 – Molecular mechanisms of α7-nAChR following agonist stimulation from Ren and colleagues' study on the protective effects of the α7R [15]. The figure traces the effects of JAK2/STAT3 signaling on anti-inflammatory actions via inhibition of NF-κβ, an inducer of inflammatory cytokine release. Potential activation of Akt as cytokine inducer is demonstrated here, however, mechanisms within pancreatic β-cells are suspected to show pro-survival/growth signaling with activation of Akt rather than attenuation of inflammatory signaling *(Gupta et al., 2018)*.

Exploring this functional role of α7-nAChR within β-cells, Gupta et al. [26] have identified increases in pro-survival signaling and anti-inflammatory actions using the full α7 agonist PNU-282987 *in vitro* in rat insulinoma INS-1 cells as well as in mice with experimentally induced diabetes. Although the results from the Gupta et. al. study set the stage for α7R mediated reductions in inflammation, PNU-282987 is not classified as human safe due to serious off-target effects found early during clinical trials [27]. Nonetheless, promising results from this experiment warrant continued exploration of this pathway as a means for restoring β-cell survival in an *in vitro* setting with induced inflammation mimicking a physiological T1D

environment and so here we will test the hypothesis that use of the human safe partial α7 agonist GTS-21 will show comparable effectiveness in anti-inflammatory/pro-survival signaling compared to the more potent PNU compound (**Figure 3**).

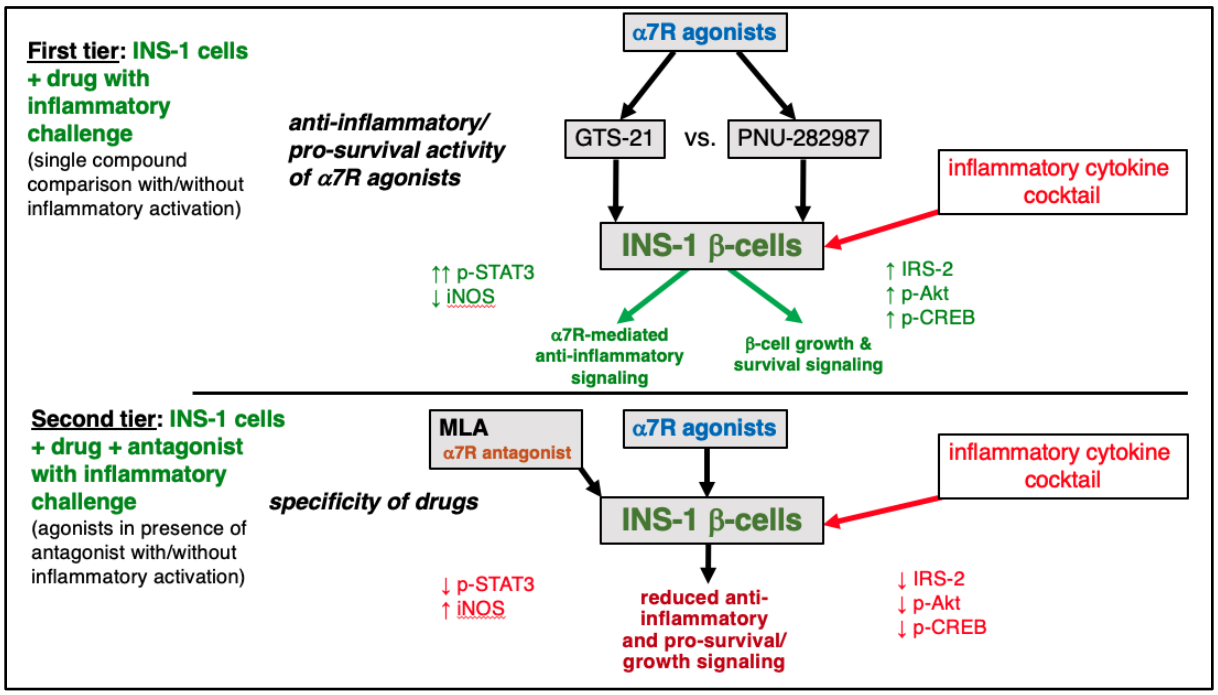

Figure 3 - Overarching scheme of experimental design for testing effectiveness of α7-nAChR agonist GTS-21 vs. PNU-282987. First-tier experiments focus on comparisons of compounds for relative anti-inflammatory/pro-survival signaling activity during an experimental inflammatory stimulus *in vitro*. Key markers of anti-inflammatory action are changes in phospho-STAT with reduced iNOS generation. Important markers of pro-survival signaling include increases in phospho- (activated)-Akt or CREB, and Irs-2. Second tier experiments will validate the specificity of agonist action with use of α7-nAChR antagonist (MLA).

Methods:

INS-1 Cell Maintenance

INS-1 (832/13) rat insulinoma cells, a very well characterized β-cell line, were maintained in standard tissue culture: RPMI-1640, 10% fetal bovine serum, 8.3 mM glucose, 10 mM HEPES, 100 mM L-glutamine, 50 mM sodium pyruvate, 100 units/ml penicillin, 100 g/ml streptomycin, and 50 μM mercaptoethanol at 37^{o}C in 95% air/5 % CO_2 (Gupta et al. 2018) using standard sterile procedures. Cells were grown in maintenance T-75 flasks and checked every 2-3 days to ensure they remained healthy. To increase cell growth within maintenance flasks for desired 90% confluency, the addition of 5 mL of complete media to previous media was done for cells under 20% confluency or 10 mL PBS washes followed by replenishing with ~15 mL complete media for cells at greater than 20% confluency. Increasing the passage number or controlling the proliferation rate was done using 10 mL PBS washes followed by 2 mL of warmed trypsin and then incubating at 37°C for 2 min. Once cells were no longer adherent to the flask surface, 2 mL of media + free floating cells were transferred to a new T-75 flask to increase passage number for long-term propagation.

INS-1 Cell Preparation for Experiments on Drug Anti-inflammatory Activity

In preparation for all *in vitro* experiments, cells within the T-75 maintenance flasks were allowed to grow to their ideal (~90%) confluency. Once cells were expanded to this stage and were ready for plating, 4x 6-well tissue culture grade plates were labeled and the media was aspirated. T-75 flasks were rinsed with 10 mL phosphate buffered saline (PBS) followed by 2 mL of warmed trypsin with a 2 min incubation. After incubation, an additional 24 mL of complete media was added to the trypsin and then 2 mL of suspended cells were distributed to a

single well among one of the 24 wells among the 4x 6-well plates labelled for experimentation. Cells were then allowed to grow for 1-3 days until reaching between 70% - 80% confluency for experimentation [28]. Following plating and growing, cells were imaged on a Nikon Ti-Eclipse microscope workstation using NIS Elements software to precisely measure cell confluency (**Figure 4**). Once an average cell confluency was determined for each plate, plates were immediately moved to the biosafety cabinet in order to begin pre-treatment manipulations.

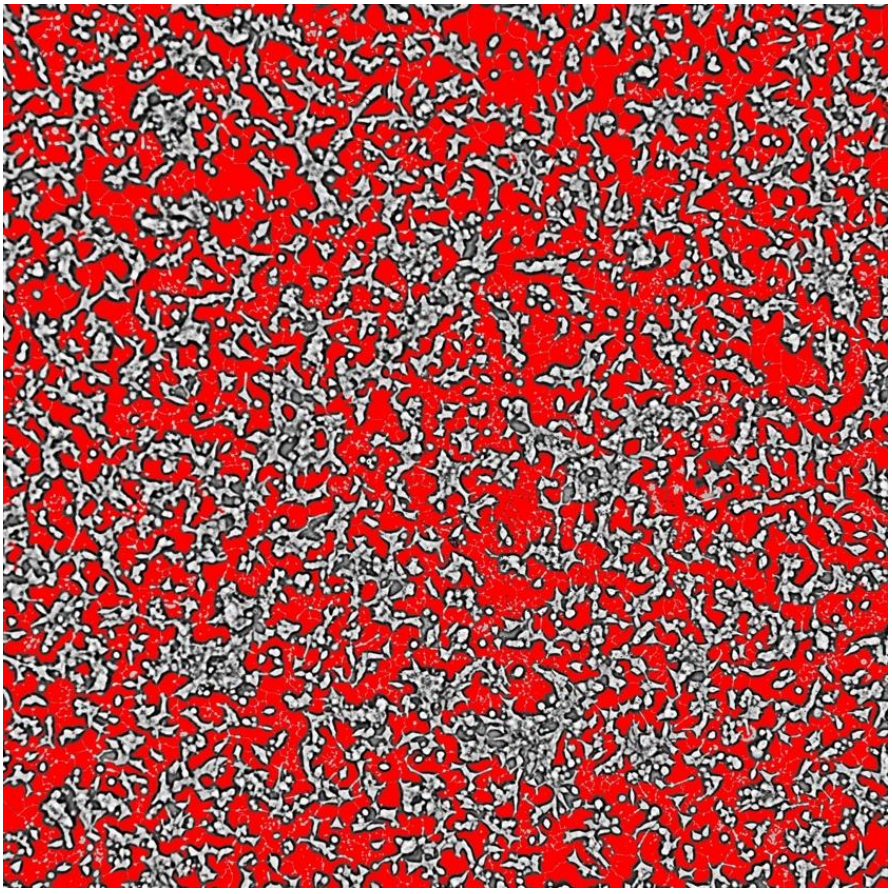

Figure 4 – Representative image from microscope workstation using NIS Elements software to measure cell confluency before use in experimentation. Calculations of density were used by measuring the area of space unoccupied by cells using a binary filling option based on opacity and then subtracting the percentage of area from 100%. This segment of cells was calculated to 73.6% confluency.

Key Reagents and Equipment

The partial agonist GTS-21 (Tocris Bioscience) and the specific agonist PNU-282987 (Alomone Labs) were used for activating α7R. The specific α7R antagonist methyllycaconitine (MLA; Tocris) was used to confirm specificity of α7R activation within experiments. The 1.0x cytokine cocktail mixture to elicit an experimental inflammatory stimulus (human IL-1ß, TNFα and IFNγ) [29] was generated from a 1000x stock solution (5 μg/ml -10 μg/ml-100 μg/ml, respectively; ProSpec Technologies). For semi-quantitative multiplex fluorescence immunoblotting, the pre-dyed molecular weights standard was obtained from LI-COR Biosciences (Chameleon Duo). Primary antibodies were procured from Cell Signaling Technologies, and secondary antibodies (AlexaFluor 680 and 790-coupled "ML" grade) were procured from Jackson Immunoresearch. The gel apparatus, the running and transfer buffers (10X stocks), EveryBlot blocking buffer used for all immunostaining experiments, and "LF" PVDF membranes used were all purchased from BioRad Laboratories. A LI-COR Odyssey imaging workstation and software (V.3.0) was used for all membrane imaging and quantitative analyses.

INS-1 Cell Preparation and Reproducibility for Experiments on Drug Anti-inflammatory Activity

In preparation for all experiments (**Figure 5**), wells were pre-labeled with their respective treatment groups. All experiments were conducted minimally in triplicate to account for a sufficient quantity of biological replicates (n = 3) and to ensure quality control of the results as well as validate later statistical results. As such, each replicate experiment was conducted with a different batch (plated from a different culture of cells within a T-75) to validate the consistency of all results observed. Furthermore, pre-determined volumes of DMSO (vehicle for drugs)

present in both agonistic treatment groups and vehicle control groups were kept to below 0.1%, the threshold for ensuring safe concentrations, since DMSO cytotoxicity can arise from levels at 1% or greater [30].

Experimental Treatments with α7R Modulators

For all experiments, cells were pretreated (1 h) with DMSO in the standard RPMI base complete media but with 10% heat inactivated fetal bovine serum (HIS). Agonist treatment groups were devised using an optimized concentration of the α7R agonists GTS-21 (150 μM) or PNU-28297 (100 μM) determined via previous optimization experiments [26]. After pre-treatment, cells were rinsed with warm PBS before introduction of the agonist treatment with or without cytokines. This standard inflammatory stimulus was induced via a 4 h incubation with a "1x" cytokine mixture in HIS–supplemented media amongst the combinations of agonists or the DMSO vehicle control. At the end of the 4 h incubation, the media was replaced with ice-cold PBS for 10 min. During this time, cell lysis buffer was prepared (ThermoFisher RIPA buffer base + protease and phosphatase inhibitors) and 250 μL of the buffer was added to each well followed by mechanical disruption of cells using a Teflon cell scraper. Each lysate was collected and then sonicated on ice for 10 sec and then centrifuged at 4°C at 15,000 rpm for 20 min before collection of remaining soluble lysate. Aliquots of 40 μL were collected from the centrifuged lysate and were labelled according to each well's designated number. All aliquoted samples were then stored in -80°C for later analysis. Finally, protein concentration was determined with the Bradford microwell assay (Bio-Rad) using BSA as a standard.

SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Standard procedures were used to cast and run 7.5% SDS-PAGE gels (Bio-Rad equipment and protocols) with 15 wells and 1.0 mm thickness. Gels had the combs removed, topped with 1x Tris-Glycine/SDS running buffer, and stored at 4°C for no more than 24 h before use.

From the measured concentrations of each lysate, each well was loaded with 15 μg of each sample. Because the protein concentrations were determined to be ~ 2 μg/μL, a 2x Laemmli sample buffer (Bio-Rad) + 5% β-mercaptoethanol was used to denature the sample. Molecular weight standards used consisted of Chameleon Duo Ladder (LI-COR). Gels were run under standard conditions using Bio-Rad equipment and reagents. While the gels were running, Low Fluorescence (LF)-PVDF membranes (Bio-Rad) were soaked in methanol and then allowed to equilibrate in 1X Towbin's transfer buffer solution (Tris/glycine/methanol). After the gels had run to completion, they were allowed to sit for about 5 min in transfer buffer. After both the gel and PVDF membrane were equilibrated, the proteins were electrophoretically "wet-transferred" at 100V for 1 h. During 1 h transfer, the presence of both an ice pack and stir bar were used to prevent overheating. At the termination of transfer, gels were Coomassie-stained followed by destaining and rehydration in water before gel drying and imaging for reference.

Analysis of α7R Activation via Anti-Inflammatory and Survival Signaling

PVDF membranes were subsequently allowed to air dry for 5 min before rehydration in methanol. After rehydrating the membranes they were subjected to a 5 min wash in 1X Tris buffered saline (TBS) and then blocked for 15 min in EveryBlot blocking buffer (Bio-Rad) before the addition of specific antibody probes. Importantly, pilot studies to confirm specificity

and establish sensitivity of each primary antibody were conducted initially before embarking on multiplex labelling studies. Probes for inflammatory signaling (phospho-STAT3/ total STAT3 and iNOS) and growth/survival signaling (Irs2, phospho-S^{473}Akt/total Akt, phospho-CREB/total CREB), and actin for standardization of bands were used at the vendor's suggested dilutions as detailed *(Gupta et al., 2018)* [26] using simultaneous near IR/IR multiplex fluorescence detection (ML-grade donkey anti-rabbit-AlexiFluor 790 and donkey anti-mouse-AlexiFluor 680 for the 700 nm and 800 nm channel detection, respectively (LI-COR Odyssey workstation) and the on-board quantitation software (Odyssey 3.0) for analysis of band molecular weight and signal intensity. Signal quantifications were conducted using Odyssey software's integrative intensity "kilo counts" via a 16-bit depth intensity scale. All integrated intensities from each band were then exported to an Excel file, adjoined to their respective treatment group, and then the relative values of each were compiled into an organized table. Comparisons of intensities against vehicle control or actin bands were done as a means of normalizing the relative levels of protein signal. Percent phosphorylation of STAT3 and Akt were done by dividing the relative signal for phosphorylated STAT3 or Akt by its total unphosphorylated counterpart. For quality control of all results, each analytical run of a sample of lysate was done in triplicate ($n = 3$) to produce technical replicates for comparison. Each treatment group was compared to its other replicate groups as a safeguard for any potential errors occurring in the analytical process. All calculated values in Excel were then exported to GraphPad Prism (v9.0) for statistical analyses using a one-way ANOVA (using a Dunnett's, Tukey-Kramer, or Student's t-test where appropriate) to denote any differences between the calculated intensity values using an alpha level of $\alpha < 0.05$ as a marker for significant statistical differences. Reproducibly was ensured using 3 biological replicates (different cell batches conducted on different days, each with 3 technical replicates).

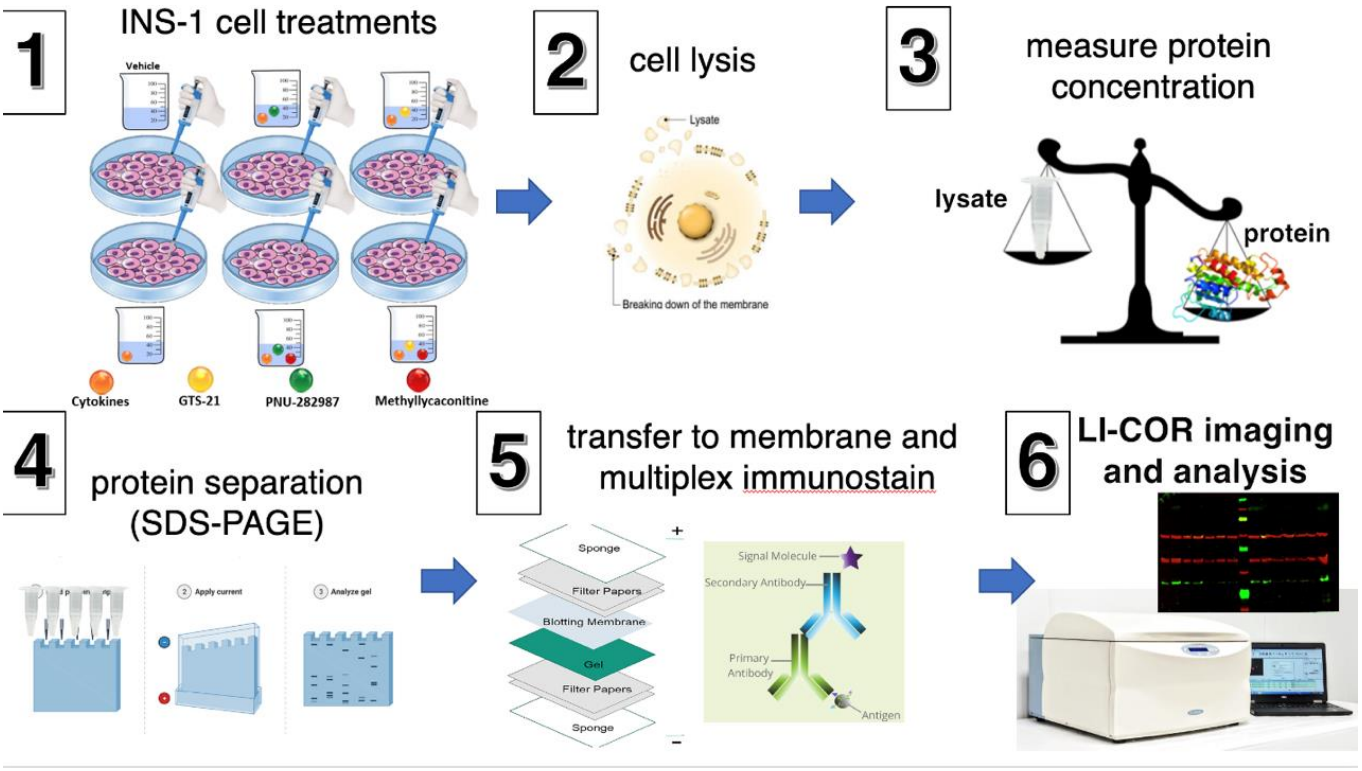

Figure 5 – Flow diagram depicting entire experimental to analytical process. Starting with growing cells and treating with drugs and pro-inflammatory reagents **(1),** then cell lysis, collection, and preparation for analysis **(2).** Next was measuring protein concentration **(3)** in order to properly load equivalent protein for SDS-PAGE **(4)** and subsequent transfer to PVDF membranes, multiplex immunostaining **(5)**, followed by fluorescence imaging and quantitative analysis **(6).**

Results:

Determination of Optimal GTS-21 Concentration

Previous experimentation by Gupta and colleagues utilized an agonist concentration of 100 μM for PNU-282987 with indications of pro-survival, growth, and anti-inflammatory signaling INS-1 cells stimulated with pro-inflammatory cytokines [26]. Being a partial agonist for the α7-nAChR, GTS-21 was initially tested in a range of concentrations from 10μM – 200μM and readouts of relative inflammatory or pro-survival signaling were used to determine its optimal concentration. At the highest concentration of GTS-21 (**Figure 6**), a reduction in activated (phosphorylated) STAT3 was observed, suggesting reduced downstream activation of the pro-inflammatory transcription factor NF-κB, potentially correlating with decreased anti-inflammatory drive [31]. However, unexpectedly, significantly reduced iNOS or increased Akt activation [32] were not observed under these conditions (**Figure 6**). Nonetheless, since 200 μM GTS-21 reduced STAT3 activation in the presence of inflammatory cytokines relative to that of the vehicle control, we opted to use 150 μM GTS-21 in subsequent experiments.

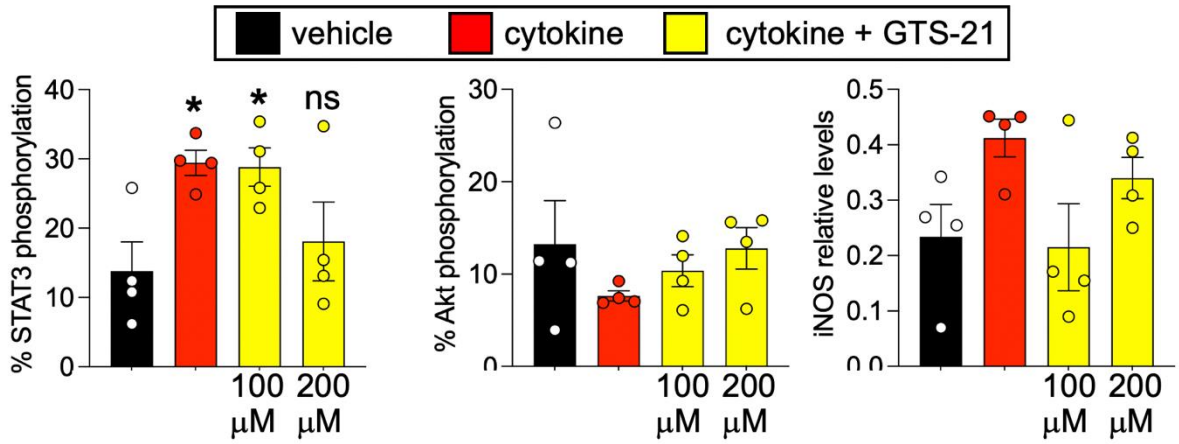

Figure 6 – Pilot study to deduce optimized concentration of GTS-21 with respect to anti-inflammatory and pro-survival signaling. Disparate results between 100 μM and 200 μM warranted further testing of GTS-21 at 150 μM in order to examine anti-inflammatory and pro-survival signaling activity. *P<0.05; one-way ANOVA with vehicle (control) as reference.

INS-1 Cells Exhibit Anti-Inflammatory Drive via GTS-21 Agonist Action at the α7-nAChR

The mechanistic role of α7-nAChR in the inflammation cascade is not completely understood; however, studies have demonstrated reduced phosphorylated STAT3 as an indication of slowed disease progression with corresponding increases in phosphorylated Akt [15]. Key proximal signaling components such as JAK2/STAT3 in the inflammatory pathway and increased survival signaling through phosphoinositide 3-kinase (PI3K)/Akt pathway have been shown to respond to agonist action at the α7R [26]. In response to the addition of GTS-21 to INS-1 cells in the presence of an inflammatory stimulus, a significant (~50%) reduction in p-STAT3 was observed when compared to cytokine control group (**Figure 7**). This decrease in p-STAT3 signaling suggests a potential direct protective action of GTS-21 via β-cell α7R is likely leading to downstream reductions in NF-κB [33], and eventually leading decreased iNOS, (**Figure 7)** within the GTS-21 treated groups. PNU-282987, previously determined to cause dose-dependent changes in p-STAT3 levels, had no significant anti-inflammatory activity at the concentrations of cytokines used. GTS-21 exhibited not only significant reductions in inflammatory signaling compared to the cytokine control groups, but also demonstrated greater anti-inflammatory action than PNU-282987 which has been previously demonstrated to have significant effects on inflammatory signaling [26].

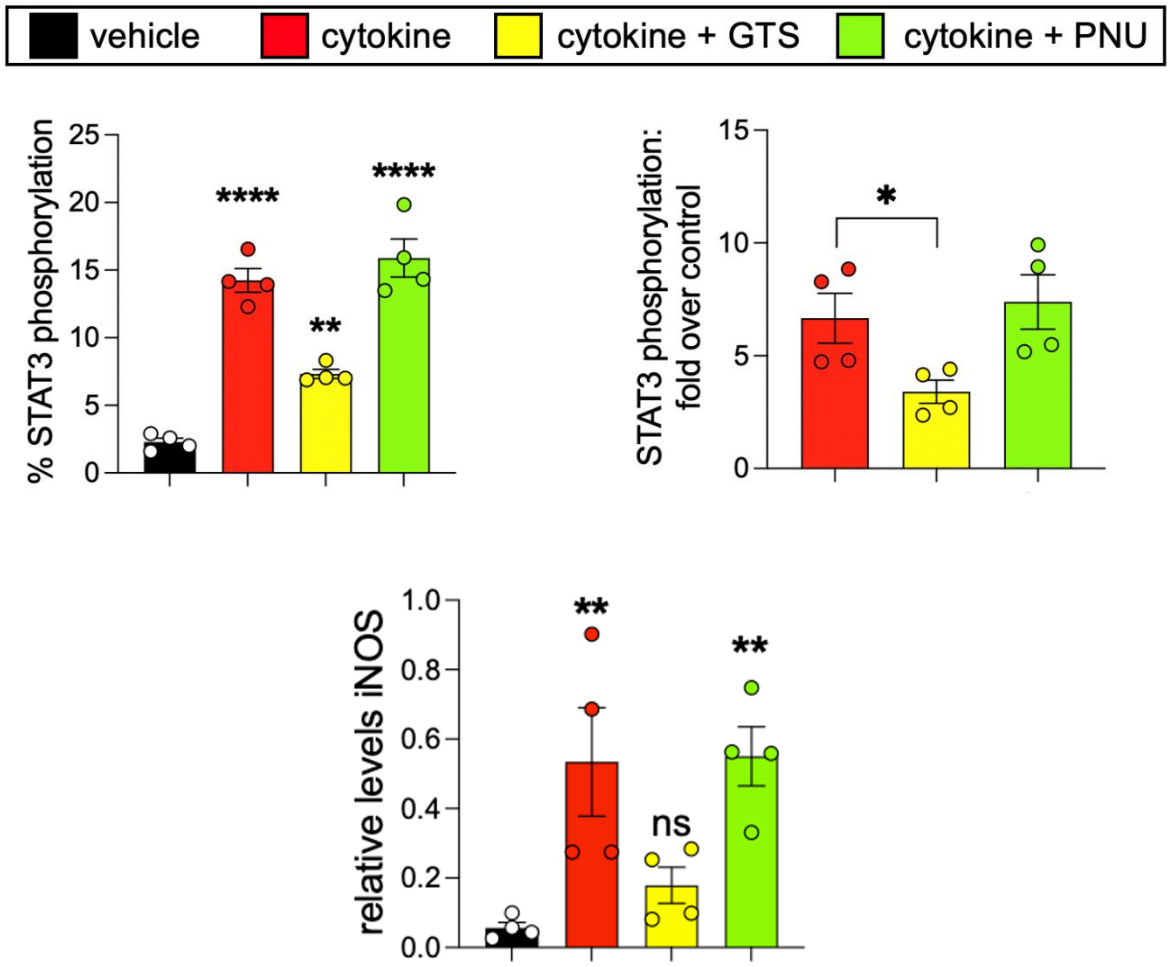

Figure 7 – GTS-21 exhibits anti-inflammatory activity. Both reductions in p-STAT3 (upper panels) and iNOS (lower panel) compared to cytokine group alone demonstrate marked anti-inflammatory drive with 150 μM GTS-21. P-STAT3 levels are reduced ~50% in GTS-21 treated INS-1 cells (upper right panel). GTS-21 restored iNOS levels to that of the vehicle control group (lower panel). Unexpectedly, under these conditions, PNU-282987 did not exhibit any anti-inflammatory activity in INS-1 beta cells. **P*<0.05 (t-test); ***P*<0.005; *****P*<0.0001, relative to vehicle control; one-way ANOVA.

INS-1 Cells Exhibit Akt Driven Pro-Survival Signaling through GTS-21 α7R Activation

Previous characterization of the α7R as a target in neurodegenerative disorders has shown the importance of pro-survival signaling through PI3K/Akt pathways [34]. A similar mechanism of action was proposed in a T1D model of β-cell survival whereby the PI3K/Akt

pathway may be exploited to halt T1D disease progression [35] and maintain β-cell mass and function. Considering the previous observation by Gupta et.al. [26], whereby β-cell Akt activation was observed in response to the full agonist PNU-282987 in the presence of pro-inflammatory cytokines, we expected both GTS-21 and the PNU compound to exhibit pro-survival activity by increased Akt phosphorylation. Accordingly, although GTS-21 treated INS-1 β-cells led to restored activated Akt levels upon cytokine treatment (>2-fold increased) (**Figure 8**), no such pro-survival effect was noted in the PNU-treated cultures. Hence, restoration of activated Akt levels to that of vehicle control in the GTS-21 treated group, but not in the PNU compound, suggest that this established human-safe compound may be suitable for further validation studies *in vitro* in human islets and, eventually, in the preclinical studies in mice.

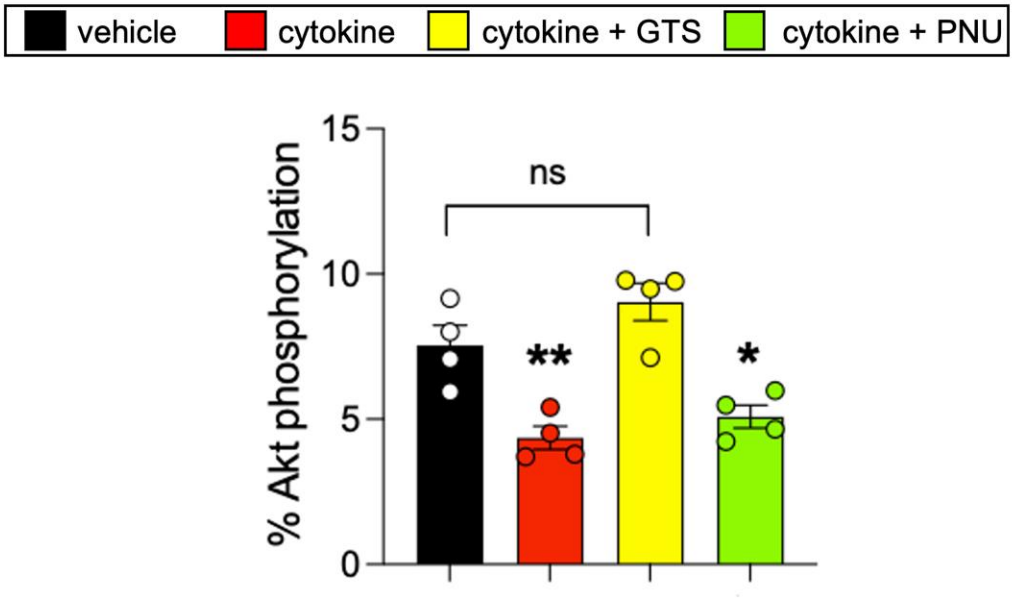

Figure 8 – GTS-21 exhibits pro-survival activity in INS-1 beta cells. GTS + cytokine-treated cells exhibit activated Akt levels comparable to that of the vehicle control group Unexpectedly, the PNU compound showed no Akt-driven pro-survival activity. ***P*<0.005; **P*<0.05; one-way ANOVA.

Discussion:

T1D progression, characterized by the immune-mediated destruction of pancreatic β-cells, is known to be perpetuated by dysfunctional autonomic signaling via the vagus nerve [11] and continued release of macrophage recruiting cytokines [36]. Both of these symptomatic ramifications of the disease are now known not to be mutually exclusive and thus, through α7R activation via specific agonists, downstream cytokine induced inflammation may be restored to normal levels. The direct mechanisms following α7R agonist activation have not been fully elucidated; however, many pre-clinical experiments have pointed to the potential efficacy of targeting the α7R in various inflammatory diseases [10,23,25].

Previous work surrounding cytokine stimulation in general macrophages was shown to up-regulate the α7R and evoke counter-inflammatory pathways [37]. Knowledge of these effects lead to a cascade of experiments working on characterizing the role of functional α7R present in various peritoneal organs. The role of the vagus nerve in abdominal organs where α7R is commonly located, such as the liver with regard to its regenerative abilities, further reaffirms its role in protecting from disease progression and cell apoptosis mediated through α7R stimulation [38]. As another example, administration of nicotine or GTS-21 in rat kidney after burn damage ameliorates ischemia-reperfusion injuries [15]. Both of these experiments point to potential benefits of α7R activation as a promising potential therapeutic strategy for T1D treatment as they potentiate the restorative capabilities of the α7R. Evidence of these beneficial effects are also seen in the α7R mediated block in NF-κB activation, thought to be a result of JAK2/STAT3 upstream activation [39]. *In vitro* and *in vivo* experimentation in mouse models focusing on brain microglial cells demonstrated suppression of NF- κB and upregulation of the Nrf2/HO-1,

upstream signaling molecules in regulating the antioxidant response, after stimulation of the α7R [40]. Further experimentation on brain astrocytes showed similar mechanistic properties of α7R activation leading to upregulation of AMPK, CREB, and PPARχ while reducing pro-inflammatory cytokines [41]. GTS-21, originally designed for use in neurodegenerative disorders involving these cell types, showed strong promise as a candidate for protecting against neuronal inflammation because of its effects on these anti-inflammatory and pro-survival pathways. GTS-21's human safe profile and minimal off target effects warranted the continued testing of α7R agonists which have now been studied in tissue specific macrophages [37], kidneys [15,42], lungs [43], liver [38], and the pancreas [26] which all consistently demonstrated NF-κB downregulation, known to be mediated by STAT3 activation within β-cells, further validating GTS-21 as a potential therapeutic candidate for inflammatory diseases. Here we demonstrated (**Figures 7 and 8**) that GTS-21 activation of INS-1 cell α7Rs supports the notion that β-cell α7R is at least partially responsible for the anti-inflammatory/pro-survival signaling we have before observed *in vivo* under an experimental pancreatic islet inflammatory environment [26]; and besides pancreatic macrophages, the β-cells themselves are also targeted by systemic α7R agonist treatment. The activation of pancreatic macrophages in unison with β-cell α7R activation may provide an amplified anti-inflammatory response based on experimentally observed anti-inflammatory effects within macrophages (**Figure 9)**.

Increases in insulin sensitivity after nicotine and PNU-282987 stimulation of α7R in normal rats and nonobese diabetic (NOD) mice through the JAK2/STAT3 [44] pathway serve as supportive evidence for the potential benefits of α7R agonists in treating T1D. Further investigation of the direct actions of GTS-21 on the functional β-cell α7R still warrant continued

exploration, but strong evidence suggests a major role of the autonomic nervous system in proper function, health, and maintenance of pancreatic β-cells [45]. Several mouse models examining the agonistic effects of nicotine or PNU-282987 on the α7R have shown prevention of T1D or T2D via non-specific cholinergic system activation. Recently, other studies have corroborated these anti-inflammatory actions of α7R in inflammatory conditions such as immune-mediated psoriasis [33] to more complex inflammatory pathways described in depression [46], a potential complication associated T1D/T2D [47,48]. Utilization of GTS-21 and PNU-282987 as α7R agonists has been shown to be effective preclinically in reducing immune-mediated inflammation in lung tissue thereby qualifying its purported effect in anti-inflammatory signaling [43]. This study sought to reproduce these intriguing results with the human safe partial agonist GTS-21 in a clonal rat β-cell line (INS-1) as a means to validate JAK2/STAT3/NF-κβ and PI3K/Akt/CREB as pathways for potential T1D disease regression when moved to a human *ex vivo* model (i.e., islets).

GTS-21 as an agonist to α7R has been known for many years; however, direct action on the JAK2/STAT3/NF-κβ and PI3K/Akt/CREB pathways through β-cell α7R within INS-1 cells still needs further validation. Utilization of the antagonists methyllycaconitine (MLA) and/or α-bungarotoxin, two specific α7R antagonists, will serve as the *bona fide* method to validate GTS-21's actions. However, both antagonists have differing degrees of effectiveness when used on various clonal animal cell lines [39]. As such, validation at the mRNA level [26] for *Chrna7* knockout (gene encoding α7R) and STAT3 knockout may be more amiable approaches for unraveling the mechanistic details of downstream α7R activation in a T1D model. A more in depth look into the specific roles of CREB as a player in survival signaling [26], and NF-κB as the

intermediary molecule between activated STAT3 and eventual iNOS activation [49], experimental α7R activation may provide further insight into GTS-21's potential translation to a human model. Current T1D therapeutics in the pipeline are still mainly focused on preventing/blocking the inflammatory cascade from perpetuating the disease; however, the actions of the α7R agonist GTS-21 as shown in the current study serve as precedent for its potential benefits for inducing both anti-inflammatory and pro-survival responses in order to retain functional β-cell mass and prevent T1D progression.

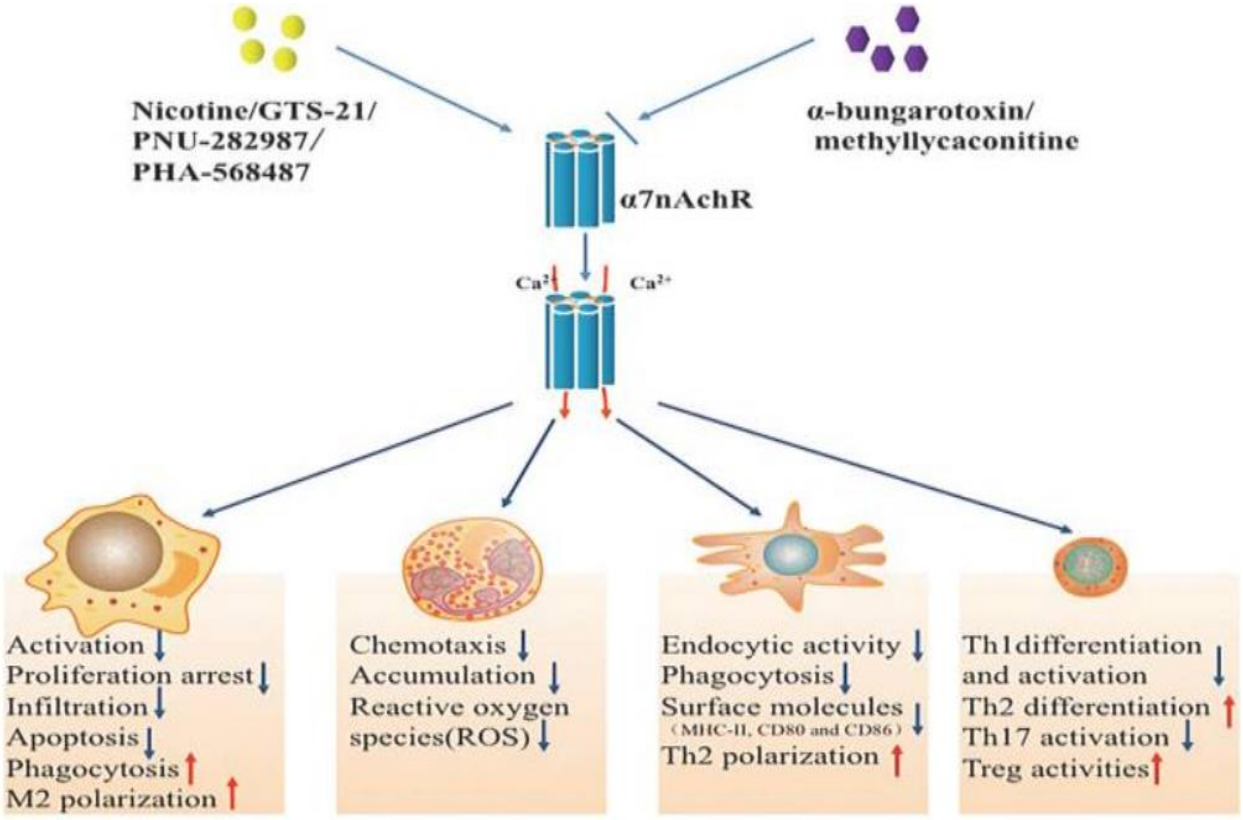

Figure 9 – Known downstream effects of α7R within the various immune cells of the body after stimulation with an α7R agonist. Overall effects of stimulation demonstrate the reduced inflammatory actions by immune cells and considerable decreases in apoptotic events indicating activation of pro-survival pathways [15].

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