Knockdown of STAT3 Protein Attenuates the Anti-Inflammatory Effect of α7-nicotinic Acetylcholine Receptor Activation in INS-1 Cells

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**Background**

The hallmark of Type 1 diabetes mellitus (T1DM) is the immune mediated destruction of insulin producing β-cells (1-3). A complex interplay between β-cells and immune cells results in the local release of pro-inflammatory cytokines such as IL-1β, TNF-α and IFN-γ, which trigger NF-κB mediated regulation of iNOS (4). Chronically induced iNOS increases nitric oxide (NO) generation, thus causing β-cell dysfunction and apoptosis (5-7). (Fig. 1)

An attractive strategy for treating T1DM would be to identify counter-inflammatory pathways in β-cells as a means of preserving islet function and survival in the face of inflammatory challenge. The autonomic nervous system via the vagus nerve curtails innate immune system over-activity in order to maintain homeostasis in response to a range of perturbations and disease states (8-10). The central cellular component of this system is the α7-nicotinic acetylcholine receptor (α7AChr), a class of ligand gated ion channels that is widely expressed in the brain, autonomic ganglia, reticuloendothelial system and pancreatic β-cells (11-14). Preliminary data suggest that β-cell α7AChR activation blocks NF-κB mediated transcription of iNOS through the activation of the transcription factor STAT3. (Fig. 1)

We attempted to elucidate the role played by STAT3 on the β-cell response to inflammation by establishing a short hairpin RNA (shRNA) mediated knockdown (KD) of STAT3 in an INS-1 cell line. shRNA is a method of silencing gene expression at the message level whereby a silencing trigger is recognized by an RNase III family nuclease called Dicer. Dicer then cleaves the trigger into shRNA that are loaded into the RNA-induced silencing complex (RISC), which uses the shRNA as a template for recognizing and destroying complementary mRNA (15). By evaluating the response of these cells to a cytokine challenge with and without treatment with the α7AChR agonist PNU, we hoped to show that STAT3 is a key player in countering inflammatory processes in β-cells.

**Experimental Approach**

**Purification of STAT3 shRNA Plasmid:** Four STAT3 shRNA constructs, along with a scramble control, were obtained from OriGene. Competent bacterial cells were subjected to heat shock in the presence of 200 ng STAT3 shRNA vector. These cells were grown in kanamycin medium and subsequently lysed to allow plasmid isolation by Qiagen midi prep kit by standard procedures. The plasmid DNA yield and quality was verified by Nano-drop and agarose gel.

**Transient transfection of INS-1 cells with STAT3 shRNA cassette:** INS-1 cells seeded in 6-well format were transfected using lipofectamine and 4.0 μg purified individual STAT3 shRNA cassette or 1.0 μg of each of the pooled four plasmid DNA cassettes simultaneously along with a control group transfected with 4.0 μg scramble control plasmid DNA. These cells were lysed and harvested after 24, 48, 72, or 96 hours. The cell lysates were screened by western blot to determine which time course produced the most successful knockdown of STAT3 protein levels.

**Stable Transfection of INS-1 cells with STAT3 shRNA cassette:** INS-1 cells seeded in 100 mM plates were transfected using lipofectamine and 10 μg purified STAT3 shRNA plasmid DNA cassette individually or 2.5 μg of each of the pooled four cassettes shRNA plasmid DNA simultaneously. The control INS-1 cells received 10 μg scramble shRNA plasmid control. The transfections were carried out in OPTIMEM reduced serum medium (Invitrogen) for six hours, at which point cells were washed with PBS and transferred to complete INS-1 cell culture medium and allowed to grow for five days. On the fifth day, transfected INS-1 cells were put on selection pressure with 2.5 μg/mL puromycin to isolate positive INS-1 clones expressing STAT3 shRNA or scrambled shRNA. We screened approximately 20 clones for stable knockdown of STAT3 by western blot screening.

**Evaluation of the Cytokines (TNF-alpha, IFN-gamma, IL-1beta) Mediated Inflammatory Response in STAT3 Knock Down in INS-1 Cells:** The cytokine challenge experiments were performed in heat-inactivated serum medium. The stable STAT3 knockdown and scrambled control INS-1 cells were pre-treated for 1 hour with vehicle (DMSO), α7AChR agonist (PNU, 60 μM), or α7AChR antagonist (methyllycaconitine (MLA),
100nM) followed by cytokine 1x cocktail (TNFα, IL-1β, IFNγ 1000x cocktail containing 5μg/mL IL-1; 10μg/mL TNFα, 100μg/mL IFNγ) for 5-hrs. The cell lysates were collected and screened by western blot analysis for total and phosphorylated STAT3, iNOS, phospho-65 NFκB and β-actin.

Results and Discussion

Transient transfection did not produce cell colonies with effective knockdown of STAT3. There were no discernible differences between the 24, 48, 72, or 96 hour time points (Fig. 2) The absence of an effective knockdown is likely due to the absence of antibiotic treatment during transient transfection. The incidence of cellular uptake of STAT3 shRNA cassette was likely low, thus requiring antibiotic selection of the cells containing the cassette to establish a useful population of cells with STAT3 knockdown.

The stable transfection produced several cell colonies with evidence of reduction in STAT3 protein levels upon western blot screening from the twenty unique cell populations transfected with STAT3 shRNA cassette. The best knockdown displayed 70-75% reduction of STAT3 protein levels. (Fig. 3) The cell population with the best knockdown had been transfected with all four of the shRNA cassettes. Only the western blot gel containing the best knockdown is presented. A green fluorescent protein (GFP) element was integrated into the cassettes, allowing transfection efficiency in the STAT3 knockdown cell population and STAT3 scramble control cell population to be evaluated by light microscopy imaging. (Fig. 4)

Previous work done by the Gupta Lab showed that rat islets pretreated for one hour in PNU serum-supplemented media with a 1x cytokine cocktail (TNFα, IL-1β, IFNγ; 1000x cocktail containing 5μg/mL IL-1; 10μg/mL TNFα; 100μg/mL IFNγ) exhibited reduced inflammatory signaling. (Fig 5) Whereas iNOS was induced 9.6±1.7-fold over control (n=3) in cytokine treated islets alone, upon α7AChR agonist PNU pretreatment, the iNOS induction signal was reduced to 3.7±0.5-fold. α7AChR antagonist MLA treatment maintained iNOS induction (9.8±1.8-fold). Phosphorylated NF-κB, correlated with increased pro-inflammatory activity, was reduced in PNU-treated islets (1.5-fold vs. 2.4-fold in cytokine only). Phospho-(T705) STAT3 correlates with anti-inflammatory signaling. Whereas in the cytokine alone treated islets pSTAT3 signal was reduced to 21% of the control islets, PNU treatment restored p-STAT3 levels to 91% of the control (cytokine treatment only), and antagonist treatment maintained reduced p-STAT3 (24% of control).

Similar experiments to those done in rat islets were performed this summer to determine the impact of STAT3 knockdown in INS-1 cells. (Fig. 6) We observed 70-75% reduction of total STAT3 and almost 50% reduction in P-STAT3 in STAT3 INS-1 knockdown clones under cytokine challenge. Scramble control INS-1 cells showed induced iNOS with cytokine treatment alone that was attenuated with the α7AChR agonist PNU, while in the STAT3 knockdown cells PNU was not as effective in curtailling iNOS induction in the face of cytokine challenge. Taken together, the islet and INS-1 cell data suggest the possibility that α7AChR agonist requires an effective STAT3 pathway to regulate iNOS expression. This project stands as additional evidence that the impact of STAT3 on inflammatory pathways in the β-cell is a promising route through which to investigate future treatment for T1DM.

Future Directions

In the limited time available for this summer project, only one population of INS-1 STAT3 knockdown cells was able to be treated with +/- cytokines and +/- PNU. Additional experimental groups must be screened to verify our data.

Further experiments need to be performed to evaluate the transcriptional interaction between STAT3 and NFκB in the regulation iNOS expression. Electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) will be used to determine the extent of the binding between NFκB and the iNOS promoter.
7. B. Maier et al., The unique hypusine modification of eIF5A promotes islet beta cell inflammation and dysfunction in mice. The Journal of clinical investigation 120, 2156 (Jun, 2010).
Pro-inflammatory cytokines such as IL-1β, TNF-α and IFN-γ cause cellular damage via the activation of NFκB, which in turn activates iNOS. Stimulation of the α7-AChR induces STAT3 to block the activity of NFκB.

The transient transfection experiment did not indicate an optimum time point for transfection. These results show (1) no significant reduction in STAT3 protein levels with respect to control and (2) variable levels of β-actin in each sample, indicating that unequal amounts of cell lysate were added to each lane.
The stable transfection experiment produced more than one cell population with significant reduction in STAT3 protein levels. The cell population with the best knockdown is presented here as STAT3 shRNA population #5. β-actin was used as an internal control.

The transfection efficiency was evaluated by establishing that the cells displayed fluorescence thanks to the GFP tag integrated into the cassette. It was determined that a significant number of cells were expressing the transfected shRNA.
Previous work done by the UVM endocrinology group in rat islets showing (1) increased inflammatory activity upon treatment with cytokines, (2) attenuation of inflammation upon treatment with both cytokines and the \(\alpha_7\)AChR agonist PNU, (3) return of inflammation to the levels seen with cytokine treatment alone when treated with cytokines and the \(\alpha_7\)AChR antagonist MLA.
STAT3 knockdown INS-1 islet cells and STAT3 scramble control cells with +/− α7AChR agonist PNU and +/− cytokine treatment show (1) decreased levels of P-STAT3 and total-STAT3 in STAT3 knockdown cells with respect to STAT3 scramble control cells and (2) less of an attenuation of iNOS activity in STAT3 knockdown cells treated with PNU and cytokines compared to STAT3 scramble control cells with the same treatment.