Award Number:
W81XWH-10-1-1055

TITLE:
Towards A Possible Therapy for Diabetes Complications

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REPORT DATE: December 2014

TYPE OF REPORT: Final

PREPARED FOR:  U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland  21702-5012

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C-peptide is the segment connecting insulin A and B chains. It is generated in pancreatic beta cells as the natural product of pro-insulin cleavage. For a long time, it was considered biologically important only for favoring pro-insulin folding within the secretory granules of the beta cells. Consistently with this view, the standard of care for diabetic, and especially T1D patients is solely insulin-replacement therapy; C-peptide is not administrated. However, recent studies have challenged this view. It has been offered increasing evidence that human C-peptide exerts intracellular effects in a variety of cells and could be of real benefit for diabetic patients who suffer from micro-vascular complications. How exactly C-peptide achieves these intracellular effects, however, is still unknown. One major advance in this area would be the identification of the specific C-peptide receptor (CPR) at the level of the cellular membrane and characterization of C-peptide/CPR signaling to effectors upon internalization.
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INTRODUCTION/ORIGINAL STATEMENT OF WORK

Diabetes mellitus is a life-threatening disease that places children (type 1) and adults (type 2) at risk of complications of blindness, kidney damage and heart disease. Diabetes afflicts 16 million Americans, with more than 800,000 new cases diagnosed each year. African, Hispanic, Native and Asian Americans are particularly susceptible to its most severe complications. Costs associated with diabetes are estimated to rich $132 billion/year. Significantly, the number of diabetes cases in the United States military mirrors national rates. Type 1 diabetes (T1D) patients lack physiological levels of insulin in their bloodstream due to the autoimmune destruction of the insulin producing pancreatic beta cells. Type 2 diabetes (T2D) patients are, instead, afflicted by an heterogeneous set of sub-syndromes characterized by peripheral insulin resistance with or without production insufficiency. Both T1D and T2D patients are at increased risk for damages of both micro- and macro-vascular tissues, which eventually bring to the well known, tragic, diabetic complications.

C-peptide is the segment connecting insulin A and B chains. It is generated in pancreatic beta cells as the natural product of pro-insulin cleavage. For a long time, it was considered biologically important only for favoring pro-insulin folding within the secretory granules of the beta cells. Consistently with this view, the standard of care for diabetic, and especially T1D patients is solely insulin-replacement therapy; C-peptide is not administrated. However, recent studies have challenged this view. It has been offered increasing evidence that human C-peptide exerts intracellular effects in a variety of cells and could be of real benefit for diabetic patients who suffer from micro-vascular complications. How exactly C-peptide achieves these intracellular effects, however, is still unknown.

In preliminary results, we have demonstrated that C-peptide reduces secretion of inflammatory cytokines from endothelial cells in a model of hyperglycemia-induced vascular injury by reducing activation of the nuclear factor (NF)-κB pathway (1). We found a similar anti-inflammatory activity of C-peptide in vascular smooth muscle cells (2). For the full-length, native, C-peptide, we found that, upon internalization from the cell surface, C-peptide quickly traffics to early endosomes and later proceeds to lysosomes for degradation (3). Trafficking of C-peptide to early endosomes is likely to account for its anti-inflammatory effects in vascular endothelial and smooth muscle cells. Based on these findings, it is hypothesized that C-peptide first binds to its cell surface receptor, then the complex internalizes and signals to effector pathways via endosomes (4). One major advance in this area would be the identification of the specific C-peptide receptor (CPR) at the level of the cellular membrane and characterization of C-peptide/CPR signaling to effectors upon internalization (5). In this project, we will set a number of experiments specifically designed to isolate the CPR and characterize its intracellular signaling activity, with the following specific aims:

1. **To isolate the C- Peptide Receptor (CPR).** We have designed and synthesized a set of biotinylated C-peptides including wild type and two mutants previously shown to not bind cellular surface membrane. These peptides will be allowed to internalize into endothelial and smooth muscle cells. Endosomes containing the biotinylated C-peptides will be isolated by cell fractionation, solubilized, and the C-peptide/CPR complexes isolated. Proteins of the wild type but not the mutant C-peptide/ receptor complexes will be sequenced.

2. **To identify endosomes as the subcellular site of C-peptide/CPR signaling.** Endosomes are likely candidate sites for intracellular signaling by the C-peptide/CPR complex. In this aim, we will block C-peptide internalization at different stages, by using pertussis toxin and endosomal Rab5 trafficking mutants, to determine at which station along its endocytic route C-peptide activates its intracellular signaling pathway.

3. **To investigate the anti-inflammatory effect of C-peptide on high glucose-induced vascular dysfunction in vivo.** We have definite proof that C-peptide displays a powerful anti-inflammatory effect on endothelial cells in vitro. It is important to investigate whether this anti-inflammatory activity of C-peptide is also observed in vivo. To this aim, we will inject C-peptide in a mouse model of diabetes-induced vascular disease and study the effect on adhesion molecule expression and macrophage accumulation particularly in the aortic segment.
Our first quarterly scientific progress report for the fourth year of our project (09/28/13 – 12/27/13) described the following:

During the course of our experiments with HSP70, we have had difficulties because of the non-specific bead-capture of HSP70 in the absence of C-peptide. While the problem was minimal with the extracellular preparations, it was frequently a problem when working with lysed cell preparations. Because the problem varied from one experiment to the next, we tested several possible causes. First, we tested if vigorous agitation of the cells or lysates during the incubation periods accentuated the problem. However, we found no change in the non-specific capture of HSP70 associated with a more vigorous agitation of the cells or lysates. We also considered that DNA released from fragile nuclei in the cell lysates might be involved. Thus, we tested the inclusion of divalent cations (Mg** or Ca**) added to the HBSS solution to help stabilize the nuclei. However, no reduction of the non-specific signal was observed.

We have also tested if different biotin capture beads may reduce the non-specific effect. In Figure 1, we compare several different beads for the signal intensity and for the non-specific signal obtained when no C-peptide was added. The High Capacity NeutrAvidin beads yielded the most intense signal followed by the High Capacity Strepavidin beads. No differences were readily apparent in the comparison of the samples captured in the absence of C-peptide with the different beads. Thus, a reduction in the non-specific signal could not be associated with any one type of bead. Because the signal obtained with the High Capacity NeutrAvidin beads was greatest compared with a nearly equal signal across all beads in the absence of C-peptide, we have used these beads for our newer experiments. The issue of this non-specific signal will be addressed further in the context of additional results later in our report.

![Figure 1. Biotin mediated capture of C-peptide-HSP70 with different beads.](image)

Several avidin beads obtained from Pierce Biotechnology were compared. HEK293 cells were treated with CP24(Biotin-HA-CP(Photoleucine at L24) and the various beads were added to aliquots of the cellular supernatant. The western blot was developed with HSP70 antibody. A parallel set of beads were treated with cellular supernatants prepared without C-peptide. In this case the intensity of the HSP70 signal was weak and approximately equal for all the different beads.

Our working hypothesis has been that the interaction of C-peptide and HSP70 involves the substrate-binding domain of HSP70. In this context, it could be expected that HSP70 will release C-peptide in the presence of ATP. Although nucleotide exchange factor facilitates this process, high concentrations of ATP have been sufficient to demonstrate the effect in its absence. We have captured HSP70 via
biotinylated C-peptide and the NeutrAvidin beads and then incubated the beads in 30mM ATP. In previous experiments we have seen a small reduction in the retained HSP70. However, in this new experiment, the HSP70 antibody signal appears slightly stronger for the ATP treated samples rather than the untreated samples (Figure 2).

Figure 2. HSP70 capture by C-peptide is not diminished by ATP.
HEK293 cells were treated with CP21(Protein-HA-CP photolucine at L21), CP24(Protein-HA-CP photolucine at L24), or CPNB (Protein-CP). The supernatant was prepared and samples captured with NeutrAvidin beads. One half of the beads of each sample were treated with 30mM ATP. The anti-HSP70 western blot shows that the signals for the ATP treated samples are slightly stronger.

We have also tested the effect of 2-phenylethynesulfonamide (PES) a molecule considered to be an inhibitor of the HSP70 substrate-binding domain. However, when PES was added to HEK293 cells before C-peptide, only a small reduction in the HSP70 signal was observed. When we used anti-hemaglutinin antigen (HA) beads for capture but used C-peptide that had only biotin attached and not the HA epitope, we observed the non-symmetrically bound signal (Figure 3) that was reported earlier in this report. A signal of the same intensity was seen with the samples which were not treated with C-peptide. However, when these samples were prepared in the presence of PES, this non-specific signal did not appear. Thus, in the case of the samples captured via the HA epitope, the reduction of signal for the PES sample appears equivalent to this loss of the non-specific signal rather than a PES mediated reduction of C-peptide-HSP70 interaction (Figure 3).

Figure 3. PES does not inhibit C-peptide – HSP70 interaction.
HEK293 cells were treated with two different C-peptides, CP21 which includes the HA epitope and CP12/24 which does not. Lysed cell preparations were UV treated and the products were capture with anti-HA beads. The HSP70 western blot shows the non-specific capture from the cells alone or the CP 12/24 samples. When phenylethynesulfonamide (PES) is included during the sample preparation, this non-specific signal is absent. During the authentic capture of the CP21 samples, the effect of PES does not appear to reduce the HSP70 any more than that which could be attributed to non-specific capture equivalent to what is seen in the other samples.
The difficulties in demonstrating reductions in the capture of HSP70 in the presence of ATP or PES may suggest that the C-peptide-HSP70 interaction is not via the currently held conventions of HSP70 and substrate interaction. Both ATP and PES affect HSP70 conformation and the lid action that is associated with substrate binding. The minimal effect of ATP and PES leads us to two possibilities. One, that the sequence of C-peptide is somewhat unique and binds to the substrate binding domain better than for most of the proteins currently known to be bound by HSP70 and may not require the added effects of HSP70 conformational changes, or two, that the site of the C-peptide interaction with HSP70 is not precisely the substrate binding domain. In the next quarter, we will identify the region of HSP70 that is cross-linked to our modified C-peptides to give support to one of these possibilities and to help guide the direction of our future investigation.

12. Use additional page(s) to present a brief statement of plans or milestones for the next quarter.

Our efforts still continue to perform experiments aimed at isolating the C-Peptide Receptor (CPR) and at acclimating the LDLR-/- mice to the high fat, high cholesterol diet before making them diabetic chemically with streptozotocin and start the C-peptide infusions again.
Our second quarterly scientific progress report for the fourth year of our project (12/28/13 – 03/27/14) described the following:

The properties of the substrate-binding site of HSP70 make this region a likely candidate for the site of interaction of C-peptide and HSP70. Because the ATP binding domain of HSP70 modulates the functional characteristics of the substrate-binding site we have previously tried to show an effect of ATP on the capture of HSP70 with our biotin tagged C-peptide. However we have only found a minimal if any effect. We now report that if our cell lysates are treated with apyrase to remove any adenine nucleotides from HSP70, there is a significant increase in the capture of HSP70 by biotin tagged C-peptide, Figure 1.

In addition, when the capturing beads were treated with apyrase there was little if any reduction in the quantity of HSP70 retained by the beads. Thus, C-peptide readily interacts with HSP70 when the ATP binding site is not occupied by adenine nucleotides and this interaction seems to be stable without additional effects mediated via the ATP binding domain. This stability may explain our previous results that did not show an effect in the presence of exogenous ATP. We can not determine from these experiments if the C-peptide binding only occurs in the nucleotide-free state of HSP70. On the other
hand, apyrase treatment might increase HSP70 interactions by promoting the release of other peptides that are more dependent on the ATP binding domain and thus making more substrate-free HSP70 available for interaction.

We also have observed that the increased capture of HSP70 in the presence of apyrase may not involve a parallel increase in UV cross-linking of the C-peptide. We are working to confirm this observation and have hypothesized that the ATP binding domain may still be involved in altering the atomic positions to enhance the possibility of cross-linking.

We have also conducted experiments to indicate that the HSP70 C-peptide interaction may affect cellular processes under conditions closer to the natural physiological state. We have previously shown that fluorescent-labeled C-peptide rapidly entered HAEC and was located in early endosomes. We have found that prior treatment of the HAEC with an HSP70 antibody blocked this entry of C-peptide while treatment with a control antibody did not interfere with C-peptide entry, Figure 2.

**Figure 2.** HSP70 antibody blocks internalization of C-peptide. Human aortic endothelial cells (HAEC) were treated with HSP70 antibody (8131), B, an isotype control antibody, C, or media alone, A. Then Alexa Fluor 488 labelled C-peptide was added to the cells. Fluorescent images of representative fields are shown in the left panel and again merged with a visible light image in the right.
In other experiments we have studied C-peptide stimulated Ca\(^{++}\) flux in RAW264.7 cells. Figure 3 shows that Ca\(^{++}\) entry as revealed by Fluro-4, a fluorescent Ca\(^{++}\) reporter, was blocked by HSP70 antibody but not blocked by an isotype control antibody.

![Figure 3](image)

**Figure 3. HSP70 antibodies block C-peptide induced Ca\(^{++}\) flux in Raw264.7 cells.** Raw264.7 cells were loaded with Fluo-4 and then treated with media containing HSP70 antibodies 8131, 810, a mouse isotype control antibody, or media alone (CP). At 30 seconds human CP was added. Both HSP70 antibodies inhibit the Increase of fluorescence corresponding to Ca\(^{++}\) entry into the cells.

Similar results were also observed with a second HSP70 antibody, (810). These two results show that the effects of C-peptide, commonly observed in HAEC and RAW264.7 cells, can be impaired by antibodies to HSP70. Thus, although many of our observations had been made in HEK293 cells which abundantly express HSP70, there appears to be a significant interaction of C-peptide and HSP70 in HAEC and in RAW264.7 cells.

12. Use additional page(s) to present a brief statement of plans or milestones for the next quarter.

We have also been attempting to identify the site of the cross-link between our photo-linkable C-peptide and HSP70. Several attempts have not succeeded, but we now believe that we have developed a technical approach that will be successful in the next quarter.
Our third quarterly scientific progress report for the fourth year of our project (03/28/14 – 06/27/14) described the following:

We have previously found that treatment of HSP70 containing lysates with apyrase to remove adenine nucleotides significantly increased the quantity of HSP70 that was captured by our biotin and HA tagged C-peptide. We tentatively concluded that the adenine nucleotides were not essential for the binding to occur or to maintain the stability of the interaction. The increased capture was attributed to a greater availability of free HSP70 because of the release of other peptides for which the binding was more dependent on adenine nucleotides.

We have now studied the cross-linking of our photolinkable C-peptide and HSP70 under these same conditions. As seen in Figure 1, the cross-linking was almost completely absent after apyrase treatment. In spite of an enhanced capture of HSP70 by C-peptide, the cross-linking is dramatically diminished.

![Figure 1. Apyrase treatment that enhances capture of HSP70 does not facilitate cross-linking.](image)

Figure 1. Apyrase treatment that enhances capture of HSP70 does not facilitate cross-linking. HEK293 cells were first lysed with 0.5% CHAPS. Lane 1 is from cells treated without, or with (lane 3), biotin-HA-C-peptide followed by capture with Neutravidin beads. Lanes 2 and 4 are like lanes 1 and 3 respectively where the beads were subsequently treated with apyrase (10U/ml x 20 minutes at room temperature) and washed before preparation for electrophoresis. In lane 5, the cell lysate was treated with apyrase before C-peptide addition and in lane 6 the C-peptide was added before apyrase treatment. This was followed by bead capture. The upper western blot was developed with HSP70 antibody. It is from our previous report and is shown here for comparison.. The lower western was developed with anti-HA antibody to reveal cross-linked, biotin-HA-tagged C-peptide. Although more HSP70 is captured in lanes 5 and 6 after apyrase treatment, there is less cross-linking than in lanes 3 and 4.

This result can be interpreted in two ways. First, there could be two HSP70 sites of interaction, one that favors cross-linking and involves adenine nucleotides, and the other less dependent on adenine nucleotides but readily able to interact with C-peptide and mediate capture of HSP70. The second possibility is that there is one binding region and that HSP70 in the absence of adenine nucleotides has a conformation that is not conducive to the formation of a cross-link. Because the ADP bound form of HSP70 is associated with a significant conformational change of HSP70, which puts the carboxy terminal domain in close proximity to the substrate-binding domain, we believe that this is the more likely explanation. Determining the location of the cross-link to HSP70 should provide additional relevant information.
We are attempting to identify the region of the cross-link formation by identification by mass spectroscopy of cyanogen bromide cleaved fragments of HSP70 which have a mass shift equivalent to the mass of the C-peptide sequence. Large quantities of C-peptide cross-linked lysates have been prepared for the capture of C-peptide cross-linked to HSP70. The samples are in the final stages of preparation for cyanogen bromide cleavage and analysis by mass spectroscopy.

This relatively stable interaction of C-peptide and HSP70 could interfere with the normal interaction of HSP70 with other peptide sequences. However, because of the abundance of HSP70 there may not be sufficient C-peptide to diminish overall HSP70 function except possibly in or near the pancreatic islets during insulin release.

12. Use additional page(s) to present a brief statement of plans or milestones for the next quarter.

In the next quarter, we plan to conclude our experiments and write a paper summarizing all the results accumulated over the period of this award.
In the fourth quarterly scientific progress report (06/28/14 - 09/27/14) of year 04, we now provide an update on our project, looking forward to completion of our tasks under a requested No-cost Extension.

On July 1, 2014, our research program re-located from the Division of Immunogenetics at the Children’s Hospital of Pittsburgh Rangos Research Center and University of Pittsburgh to the newly formed Institute of Cellular Therapeutics at the Allegheny Health Network Research Institute. While the relocation process went smoothly, we were unable to perform any work on this project during the last quarter as it took us considerable time to put the labs back into order and to become functional once again.

It is to complete our study that we requested a final no-cost extension. During this time, we intend to submit a manuscript of our findings to-date for publication.

We will also complete the sample preparation and mass spectroscopy analysis of C-peptide cross-linked with HSP70.

Furthermore, we will use apyrase treatment of purified HSP70 to investigate the interaction of HSP70 and C-peptide. We have previously obtained our best results when C-peptide was added to cells or cell lysates and not to supernatants. These results combined with the results with apyrase have led to the hypothesis that C-peptide is optimally bound to a conformation of HSP70 that is devoid of adenine nucleotides or one that may be transiently similar to this conformation. Cycling of the HSP70 conformation between the different adenine nucleotide states would explain why the more crude preparations are more successful for us. Apyrase treatment may allow us to work with a more purified HSP70 fraction to more precisely study the binding of these two molecules.

We will study the C-peptide sequence requirements for the HSP70 interaction. Many of the C-peptide responses can be partially elicited with the C-peptide carboxyl pentapeptide and we have found that the pentapeptide sequence does not block HSP70 capture with full length C-peptide. However, because this carboxyl end is short and our cross-links are nearby at positions 21 and 24, we will begin by truncating the amino terminal end. Because eliminating the terminal charged amino acids many cause significant change due to charge alone, we will remove hydrophobic amino acids near the amino terminus and determine if the HSP70 capture is diminished. The goal is to determine if a minimal C-peptide derived sequence can capture as well as the full length molecule and thus better define the region of interaction. We will also alter the C-peptide sequence by substituting amino acids near the middle of the sequence with threonine or serine to reduce the hydrophobicity to help pinpoint the region involved in the interaction. These results will better define the region of C-peptide involved in the interaction and may also be helpful for the further development of a therapeutic agent.

Finally, by using ATP and ADP affinity chromatography we will attempt to purify the C-peptide-HSP70-nucleotide complex to be used in assays of C-peptide function. We do not know if the HSP70 bound C-peptide can still cause C-peptide responses or if the binding interferes with this activity. These purified preparations will also be tested in C-peptide assays to determine if the assays can detect C-peptide bound to HSP70.
KEY RESEARCH ACCOMPLISHMENTS:

We have demonstrated an interaction between C-peptide and HSP70 that is sufficiently stable to allow the affinity capture of HSP70 with biotin or hemaglutinin antigen tagged C-peptide. The two molecules are sufficiently close to permit a zero length light activated cross-link between HSP70 and the C-peptide leucines at position 21 or at position 24 of C-peptide. Native human C-peptide and Pig C-peptide block this capture. Mouse C-peptide and the reverse sequence Human C-peptide are less effective in blocking the capture.

HSP70 capture is increased after treatment with apyrase which removes adenine nucleotides from HSP70 and possibly releases previously bound substrates of HSP70. However, cross-linking does not increase in parallel. Thus, there is a stable interaction in the absence of adenine nucleotides. But, cross-linking could occur later when HSP70 cycles through the different states of adenine nucleotide interaction that have different HSP70 conformations.

Many of our observations are possible because of the abundance of HSP70 in HEK293 cells used in our experiments. Nevertheless, this interaction of C-peptide and HSP70 may have physiological significance since we have found in cells expressing more normal amounts of HSP70 that antibodies against HSP70 block the cellular uptake of C-peptide and also block C-peptide induced calcium flux.

REPORTABLE OUTCOMES:

Rudert W A, Geng X, Fan Y, Trucco M, Human proinsulin C-peptide interacts with HSP70 (manuscript in preparation).

Rudert W A, “A Binding Partner for C-Peptide: Implications for Autoimmunity and Diabetic Complications”, Institute of Cellular Therapeutics, Work-in-Progress Seminar, Allegheny General Hospital, Pittsburgh, PA, October 2, 2014

CONCLUSION:

The previous view that C-peptide is merely an inert by-product of insulin biosynthesis seems no longer tenable. Results from studies in T1D patients and animal models demonstrate that C-peptide in replacement doses exerts beneficial effects on the early stage functional and structural abnormalities of both the kidneys and the peripheral nerves. Even a cautious evaluation of the available evidence presents the picture of a previously unrecognized bioactive peptide with therapeutic potential in an area where no causal therapy is available today. (See also Luppi P, Cifarelli V, Wahren J. C-peptide and long-term complications of diabetes. Pediatric Diabetes 2011: 12: 276–292).

Undoubtedly, the identification of the mechanism whereby C-peptide interacts with cell membranes, delineation of its intracellular signaling pathways in different cell types, and further evaluation of its transcriptional effects will enhance our understanding of C-peptide bioactivity.

On the clinical side studies of long duration (>6 months) are performed to document the robustness of its beneficial effects on the different types of long-term complications in order to define its possible role in the therapy of T1D.

Despite the fact that our knowledge is still incomplete, we believe there are several lines of evidence in support of the notion that C-peptide is a bioactive peptide and that its replacement in T1D may be beneficial in the treatment of long-term complications. Even though the nature of the peptide’s interaction with the cell membrane is only partially understood, its intracellular signaling characteristics and end effects are now well established for many cell systems not only by us but also by other valuable investigators.
Our recent results establish that C-peptide interacts with HSP70, especially as is revealed by cross-linking of the two molecules. This interaction of our n-terminal tagged C-peptide can be blocked with native C-peptide as well as C-peptides from other species. While we have only begun to explore the functional significance of the interaction of HSP70 and C-peptide, we have observed that antibodies to HSP70 reduce cellular uptake of C-peptide and reduce the calcium flux that is stimulated by C-peptide. This previously unknown interaction may have importance for better understanding the many different aspects of C-peptide and HSP70 biology.

The So What Section:

What is the implication of this research?

The possibility that the interaction of C-peptide and HSP70 may mediate or alter the effects of C-peptide is a significant concern in the study of C-peptide biology and in the development of C-peptide therapeutic interventions. Implementing a successful C-peptide treatment could easily depend on better understanding this previously unknown interaction. It is not known if assays of C-peptide detect C-peptide bound with HSP70 or if HSP70 bound C-peptide can mediate the known physiological responses to C-peptide. Likewise proinsulin, that contains the C-peptide sequence, might also bind with HSP70 which could enable immunological responses due to an exposure of insulin epitopes in a non-native conformation. In this case, C-peptide might compete with proinsulin during the interaction with HSP70 and minimize these potentially harmful immunological responses.

The modifications to C-peptide to extend the half-life of circulating C-peptide for therapeutic applications as are now in clinical trials may either increase or may lessen the interaction of C-peptide and HSP70, one of which could improve the therapeutic effectiveness of modified-C-peptide therapy.

The study of each of these possibilities could further the understanding of the role of C-peptide and the development of effective C-peptide therapy aimed at reducing the complications observed in diabetic patients being treated with insulin in the absence of C-peptide. Further investigation of these possibilities is warranted because of the potential impact on development of new therapies.

What are the military significance and public purpose of this research?

As the military is a reflection of the U.S. population, improved understanding of the underlying etio-pathology of T1D will facilitate the development of potential therapeutics to prevent the onset of the disease or the development of diabetic complications among active duty members of the military, their families, and retired military personnel. Finding a cure to T1D will provide significant healthcare savings and improved patients' well being.