

A. Statement of Hypothesis and Specific Aims:

Sepsis due to the complications of bacterial infection and endotoxin in the blood is the leading cause of death in intensive care units in the United States. The death rate from this condition ranges between 30 and 70 percent under the best of care¹. Basically, sepsis is caused by an over-reaction of the body's host defenses to severe bacterial infection². However, the mechanisms that regulate this process are largely unknown and represent a major gap in our knowledge.

This project focuses on preventing the pathology of sepsis, including disseminated intravascular coagulation (DIC), acute respiratory distress syndrome (ARDS) and multiple organ failure (MOF). Our laboratory studies a receptor called Triggering Receptor Expressed in Myeloid cells (TREM)-Like transcript-1 (TLT-1) that is exclusively found on platelets in the peripheral blood of mice and humans^{3, 4}. Platelets play an integral part in thrombin generation and clot formation, and by controlling thrombin activation, influence the outcomes of sepsis. There are several lines of evidence to suggest that TLT-1 can be used to manipulate platelets to regulate the coagulopathy leading to DIC, ARDS and MOF. First, TLT-1 is only found on platelets making it a target for controlling platelet activation. Second, we can demonstrate that engaging TLT-1 can inhibit thrombin mediated platelet activation *in vitro*, implying that it can reduce thrombin and fibrin production *in vivo*. Third, a 17 amino acid stretch of TREM family member TREM-1 with substantial structural homology to a 17 amino acid stretch found in TLT-1 greatly reduces mortality from sepsis in mice^{5, 6}. Fourth, preliminary data in TLT-1 null mice demonstrate that removal of TLT-1 exacerbates the body's response to the Shwartzman reaction, which correlates with the progression of sepsis and DIC in humans. These results have been interpreted by us and others to show that the 17 amino acids of TREM-1 may be affecting TLT-1 function. And, in fact, we can demonstrate that the 17 amino acids of TLT-1 can be used to decrease the mortality associated with sepsis. Below, we develop the hypotheses that therapeutics targeting TLT-1 can be used to improve outcomes in sepsis. **This project addresses the hypothesis that TLT-1 plays a prominent role in the progression of sepsis and can be used as a therapeutic target in the treatment of septic patients.** We will show that inhibition of TLT-1 extracellular domain/ligand interactions can be used as a treatment for sepsis alone and synergistically with the therapeutic targeting of other members of the TREM locus. Furthermore release of the extracellular domain in the plasma can be used to clinically diagnose the progression of sepsis. We have developed two specific aims to test this.

Aim 1: Pre clinically qualify therapeutics against TLT-1 and other TREM family members: We will (i) determine if peptides representing the LP17 of TLT-1 will affect progression of sepsis after cecal ligation and puncture; (ii) investigate whether targeting multiple members of the TREM locus during sepsis have synergistic effects

Aim 2: Demonstrate that TLT-1 can be used as a marker for the development of DIC, ARDS, and Sepsis. We will (i) develop an ELISA and dot blot assay to identify the TLT-1 extracellular domain in the blood; (ii) identify human cohorts suffering from various stages of sepsis, including DIC and ARDS; and evaluate them for the presence of TLT-1 in the plasma using dot blot and ELISA.

At the conclusion of this project, we will have demonstrated that TLT-1 plays a prominent role in the progression of sepsis and that TLT-1 can be used as a marker for the progression of sepsis. We will have also developed new reagents with therapeutic potential.

B. Background

The wound healing process is a delicate balance between the inflammatory and hemostatic processes. Traumatic injuries or bacterial infection can greatly alter this balance, leading to systemic activation of inflammation and coagulation^{2, 7}. Based on the current paradigm in which the inflammatory response launches coagulation processes into anarchy, it is logical to hypothesize that if we can control the "cytokine storm", we can control the outcomes in sepsis.

Centered on this principle, several clinical trials have been designed to control the inflammatory response. These include trials on Tissue Necrosis Factor (TNF)- α , the general inflammatory process, IL-1b and bacterial endotoxin. However each of these attempts have produced less than desirable results. If patients are already suffering from a coagulopathy when diagnosed with sepsis, then treatments focusing on the coagulation pathway may give more promising results. Current literature supports this hypothesis⁸ and suggest a shift in treatment regimens toward those that focus on coagulation or both inflammation and coagulation pathways.

The TREM Locus: We continue to uncover new players in the progression of the inflammatory response. A newly uncovered locus is emerging as a prominent factor in the amplification of sepsis. The triggering receptors expressed in myeloid cells (TREM) represent six genes on human chromosome six. Many of these genes are conserved in mice, making them excellent targets for study. Work by two independent laboratories has defined a role for TREM-1 in sepsis. Bouchon *et al.* has shown that a TREM-1-Fc fusion protein decreases mortality 85% in the murine endotoxemic shock model of sepsis, even after a 4-hour delay in the addition of the fusion protein⁹. Gibot *et al.* further defined this model by aligning the murine and human TREM-1 extracellular domain and identifying a stretch of 17 amino acids (LP17) that were highly homologous. They found that the addition of the LP17 peptide to mice in the endotoxin model of sepsis decreased mortality 100%, and 75% in the more stringent cecal ligation and puncture model of murine sepsis⁶. Furthermore, the presence of the TREM-1 soluble fragment shows a positive correlation with the progression of pneumonia or sepsis in patients¹⁰. These results suggest LP17 is an attractive therapeutic target in the inflammatory response during sepsis.

The final characterized member of this locus, and the focal point of this study, is called TLT-1 and, is found in mice and humans³. Our characterization of TLT-1 demonstrated that it is abundant, specific to the platelet and megakaryocyte lineage, and is sequestered in the platelet alpha granules. Upon platelet activation with thrombin or LPS, TLT-1 is translocated to the platelet surface^{4, 11}. TLT-1 contains a v-set Ig type-extracellular domain, a transmembrane region and a cytoplasmic tail that contains a membrane distal immunoreceptor tyrosine-based inhibitory motif (ITIM) and a polyproline-rich domain. It does not couple to the DAP 12 activating chain like other members of the TREM family; however, it has been shown to enhance Ca⁺⁺ signaling in rat basophilic leukemia (RBL) cells, suggesting TLT-1 is a co-activating receptor. The specificity of TLT-1 expression suggests that it plays a unique role in hemostasis and/or thrombosis^{3, 4}.

Our recent characterization of the TLT-1 extracellular domain has uncovered evidence that suggests that TLT-1 also functions during the onset and progression of sepsis^{5, 11}. First, like TREM-1, a soluble fragment of TLT-1 is identifiable in the serum of humans, although to date no correlation has been made to human disease. The second realization comes from the structural analysis of the TLT-1 extracellular domain. When the amino acids of the extracellular domain of TLT-1 and TREM-1 are aligned, the homology seen in the region of the LP17 extends to TLT-1. The crystal structures of TLT-1 and TREM 1 demonstrate the structural similarities that TLT-1 shares between the TREM family member TREM-1⁵. Residues 94–110 in hTLT-1 share considerable structural homology to mTREM-1 residues 103–119 (LP17). In both TREM-1 and TLT-1, these peptides are significantly exposed, with both ends forming exposed loops, (Figure 1). The similarity between the loops that house LP17 of TREM-1 and TLT-1 open the possibility that the TREM-1 LP17 may also improve sepsis outcomes by affecting coagulation through interactions with TLT-1 as well.

Summary: It is clear that there are many gaps in our understanding of the progression of sepsis to MOF. Attempts to intervene in the inflammatory process have met with a great deal of disappointment. More recent trials focusing on coagulation pathways have provided more success, suggesting that development of treatments for sepsis should also focus on the inhibition of coagulation processes. Furthermore, it is necessary to identify more of the key players in the progression of sepsis. Our model predicts that TLT-1 is a thrombotic molecule and that

intervention with TLT-1 may have significant impact, not only on diseases associated with sterile thrombosis, but in the management of sepsis and septic shock as well. Furthermore, targeting two or more TREM family members may act synergistically toward decreasing the mortality associated to sepsis.

C. Preliminary Studies:

This section demonstrates how we address problems in the laboratory setting, i.e., the questions asked; the rationale behind the questions; the experiments used to address the questions; the results; and the implications relative to this grant application.

Question: How does TLT-1 affect platelet function? Rationale: TLT-1 is

an abundantly expressed platelet receptor that is unique to platelets. TLT-1's restricted pattern of expression suggests a specific role in platelet function. Here, we ask if we can manipulate platelet aggregation using proprietary antibodies specific to TLT-1 or fusion proteins derived from TLT-1.

Experimental design: We examined if engagement of TLT-1 using various antibodies affect platelet aggregation. Aggregation assays were run on an aggregometer (Chrono-log Corp. Havertown PA). Antibodies or vehicle were added and allowed to incubate with the washed platelets for at least 3 min at 37°C with stirring (800 rpm) before the addition of thrombin. Thrombin was added to the aggregation cuvette containing 600 µl of platelet suspension at a final concentration of 3×10^8 /ml and results were recorded by a chart recorder. Doses of thrombin used were chosen based on optimal response from dose response curves on each patient's platelet samples. In most instances, we used 0.125 units of thrombin.

Results: We isolated monoclonal antibodies for TLT-1 using a fusion protein of the TLT-1 extracellular domain and the fc region of IgG₁ as the immunogen. We demonstrated their specificity to TLT-1 by ELISA, antigen-captured ELISA (data not shown), flow cytometry against TLT-1 transfected cells (data not shown) and purified human platelets (**Figure 2A**).

We proceeded to test whether our anti-TLT-1 would affect platelet function *in vitro*. Aggregation experiments were completed on washed platelets as described above and we used an unrelated isotype control and the vehicle phosphate buffered saline (PBS) as negative controls. Using thrombin on washed platelets, we were able to demonstrate a significant inhibition of aggregation in the presence of our antibodies (**Figure 2B**). This inhibition was not seen when using the vehicle alone or the negative clone. We are currently evaluating the effect of our antibody on platelet activation with other agonists, such as collagen and ADP.

Implications: Based on the aggregation traces, it is clear that TLT-1 mediated inhibition follows thrombin induced shape change during a period that would be described as the onset of primary aggregation. The major activation events that occur during primary aggregation include cytoskeletal rearrangements, granule secretion, Ca⁺⁺ influx, and inside-out signaling, any of which could be affected by TLT-1. Our interpretation of these data is that the scFv blocks TLT-1 from binding its ligand during platelet activation and an otherwise normal activation signal is

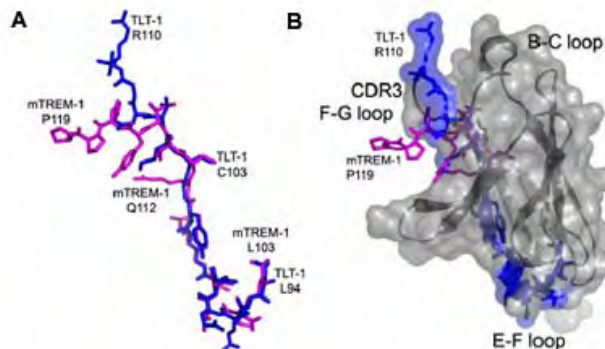


Figure 1 - Comparison of the active mTREM 17 amino acid peptide and equivalent TLT-1 residues. (A) Structural alignment of the 17 amino acid segments of mTREM-1 (residues 103–119, shown in magenta) and hTLT-1 (residues 94–110, blue). (B) Transparent molecular surface of TLT-1, shaded blue over the area of the surface formed by residues 94–110 of TLT-1. Superimposed on the TLT-1 molecule are residues 103–119 of mTREM-1 from the crystal structure (1U9K). Taken from Gattis, et. al. (see ref 3).

not being sent. Thus, TLT-1 seems to function by increasing platelets' sensitivity to thrombin stimulation.

Question: Does TLT-1 play a role in sepsis?- Rationale: The crystal structure of TLT-1 suggests that the LP-17 of TREM-1 may affect more than just TREM-1, or that TLT-1 may also be involved in sepsis as well. The standard approach for studying the function of a gene *in vivo* is to generate a null mutant. Accordingly, we targeted TLT-1 and created a TLT-1 null mouse.

Experimental Design: We chose to use a modified Bacterial Artificial Chromosome (BAC) construct to remove exons one and two of the TLT-1 gene. This completely removed protein expression of TLT-1 (**Figure 2C**). The Shwartzman model of localized vasculitis is used as a model for DIC and sepsis and would give indication of TLT-1 involvement in sepsis¹². The Shwartzman model of localized vasculitis produces a small lesion and allows us to study the necrotic area by histology. In the local reaction, the skin site was shaved and primed by a subcutaneous injection of lipopolysaccharide (LPS). Then a local hemorrhagic vasculitis was stimulated by a subcutaneous injection of PBS (control) or tissue necrosis factor (TNF)- α in the same site as the priming injection. The lesions were scored for hemorrhage, microclots, influx of neutrophils, and area of necrosis from no effect (0) to hemorrhagic necrosis (4).

Results: We have completed a initial characterization of the null mouse. We performed differential cell counts, bleeding time and aggregation curves. As expected, the difference between the null and controls is subtle within these basic parameters. There was no difference in the blood cell numbers. Bleeding times in the null mice were increased at least 30% compared to that of controls (controls - 87.33 ± 13.73 vs TLT-1 null - 184.33 ± 57.57 -12 animals) which was consistent with a decreased ability of the null platelets to aggregate compared to controls (data not shown). The major difference observed was when we addressed TLT-1's potential involvement in sepsis.

Figure 2D shows the dramatic difference in the response of the TLT-1 null mice compared to control mice in the Shwartzman reaction. The removal of TLT-1 from mice causes a larger area of necrosis (2:1), hemorrhage (3:1), more microclots containing fibrinogen and platelets (5:1), a larger influx of neutrophils to the area after exposure of LPS and TNF- α .

Implications: The inflamed area shown in **Figure 2D** demonstrates in a genetic model that TLT-1 is necessary for the body to manage its response to induced vascular leakage. Moreover, these results demonstrate that TLT-1 plays a role in managing clots when there are low levels of agonist present. These results are consistent with the increased bleeding time also seen in these preliminary studies.

Question: Can TLT-1 be targeted for therapeutic intervention of sepsis? -Rationale: The Shwartzman reaction described above demonstrates that TLT-1 is involved in the pathology of sepsis. Although TLT-1 seemed to have a slight bleeding phenotype, we believe that this delay in the ability of the platelets to aggregate will translate into slowed progression of sepsis to organ failure. Therefore, we attempted to pharmacologically use peptides to attempt to decrease the mortality associated with sepsis.

Experimental design: To evaluate the probability of TLT-1 as a therapeutic target, we used endotoxic infusion. Mice between 20-23 grams were given LPS in PBS, PBS and the TREM-1 LP17, or LPS and the TLT-1 LP17 (the equivalent amino acids (94-110) to the TREM-1 LP-17) and monitored over 168 hours. Eye bleeds were taken at 0 and 6 hours to measure TNF- α levels.

Results: The percent survival of mice, shown in **Figure 2E**, demonstrate that TLT-1 LP17 function to reduce the mortality associated with sepsis by 70% and the TREM 1 LP-17 reduced mortality by 50%. These results suggest that TLT-1 LP-17 functions as well, if not better, than the TREM-1 LP17. The TNF-a levels were measured as in ref¹³. The TNF- α levels in the TREM group, as expected, were lower than controls. The TLT-1 group unexpectedly had higher levels than control, suggesting that these two peptides may affect different mechanisms.

Implications: Here we demonstrate TLT-1 is a viable target for pharmacological intervention during sepsis. Furthermore, the difference in TNF α levels between the two experimental groups suggests that these peptides may be working via different mechanisms and in fact may be able to work synergistically. This is addressed in Aim 1

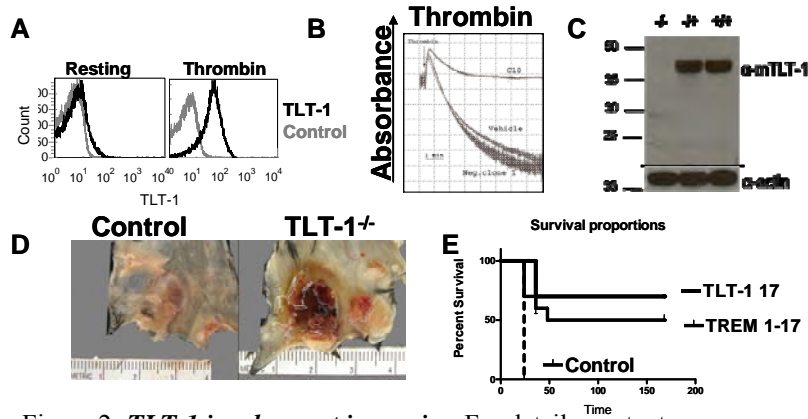


Figure 2- *TLT-1 involvement in sepsis* – For details see text.

Summary: TLT-1 is a platelet receptor that is abundantly expressed and the larger portion of the protein is found in the α -granules of resting platelets and megakaryocytes. Upon stimulation with activating substances such as thrombin and ADP, TLT-1 redistributes to the surface and throughout the platelet. In serum, a TLT-1 soluble fragment can be identified that is not present in plasma⁵. The presence of a TLT-1 fragment released

after platelet aggregation may be used as a marker for DIC and sepsis. This will be evaluated in Aim 2. In our preliminary results section, we demonstrated that a monoclonal antibody specific for TLT-1 can inhibit thrombin mediated platelet aggregation. Using a genetic model, we prescribe a role for TLT-1 in sepsis using the Shwartzman model of vasculitis and further demonstrate potential of TLT-1 as therapeutic agent using TLT-1 specific peptides. Our present interpretation of these results is that TLT-1 is a pro-thrombotic molecule that acts by facilitating platelet activation in the presence of low concentrations of agonist. Furthermore, we submit that TLT-1 mediated inhibition can be translated into a decrease in the coagulopathy associated with sepsis and is a good therapeutic target for sepsis and this is addressed in Aim 1.

D. Research Objectives and Methods:

Aim 1: Pre clinically qualify therapeutics against TLT-1 and other TREM family members:

We will (i) determine if peptides representing the LP17 of TLT-1 will affect progression of sepsis after cecal ligation and puncture; (ii) investigate whether targeting multiple members of the TREM locus during sepsis have synergistic effects;

Rationale: TLT-1 is the second member of the TREM locus that has been demonstrated to play a role in sepsis and this opens up the possibility that other members of the locus may also be factors in sepsis. Therefore, we aim to achieve a more complete understanding of the locus and whether other members of this locus may be used in combination with TLT-1 to treat sepsis.

Experimental design: (i) Determine if peptides representing the LP17 of TLT-1 will affect progression of sepsis after cecal ligation and puncture: The cecal ligation and puncture (CLP) model of resuscitated shock best reproduces the hypotension, hyperdynamic state, and degree of mortality seen in patients with septic shock¹⁴. Mice (20–23 g) will be randomly grouped and anesthetized with ketamine/xylazine and made septic with cecal ligation and punctured with an 18-gauge needle. A small amount of stool is expelled from the punctures to ensure patency. One group of control animals undergoes a similar operation, but the ligation and puncture is omitted. At the completion of this procedure, one dose of buprenorphine is administered, and following that, no further anesthesia is given. Treated mice are resuscitated with fluids (50ml/lg normal saline at the time of surgery and every 6 hours (hr) thereafter, subcutaneously). The animals will be treated with normal saline, control peptides (same amino acids expressed in a random order), and TLT-1 LP17. We will also evaluate the window after the induction of sepsis to determine whether treatment with LP17 is effective. We will treat separate groups of mice at

2, 4, 6, and 8 hours after the induction of sepsis and monitor their survival. These procedures will be completed for both the CLP and the endotoxin infusion models of sepsis. We will examine 10 mice per group.

To examine if TLT-1 affects the acute events of sepsis, we will use endotoxin infusion in mice. The endotoxin infusion model will lead to significant mortality in 12 hours. Therefore, endotoxin infusion in mice is most useful for the study of acute events in sepsis¹⁴. Endotoxin and infusion will be completed as described in the preliminary results section. We will examine 10 mice per group.

Seven additional animals per group will be sacrificed under anesthesia at 24 h after cecal ligation and puncture for the determination of bacterial count, measure of coagulation parameters (TAT complexes and D-dimers: measured by ELISA kits), and cytokine levels. Serum samples will be collected by cardiac puncture and assayed for TNF- α , IL-1 β and IL-6 by ELISA (BD Biosciences, R&D Systems) sTREM-1 and TLT-1 levels will be measured by immunodot. For the assessment of bacterial counts, blood and peritoneal lavage fluid will be plated in serial log dilutions on tryptic soy supplemented with 5% sheep blood agar plates. Peritoneal lavage fluid will be obtained using 2 mL RPMI 1640 (Life Technologies). After plating, tryptic soy agar plates will be incubated at 37°C aerobically for 24 h and anaerobically for 48 h. Results will be expressed as CFU per ml of blood and CFU per mouse for the peritoneal lavage. Our laboratory technician has considerable experience in the handling of mice and we perceive no difficulty in achieving clean interpretable results from these procedures.

(ii) Investigate whether targeting TLT-1 and TREM-1 locus during sepsis will have synergistic effects: We will use a combination of the effective dose 50 of TLT-1 and TREM-1 peptides in both the CLP and endotoxin infusion models as described in Aim 1i and preliminary results, respectively.

Expected Results: We expect TLT-1 LP17 to reduce sepsis in CLP-induced sepsis in a similar manner as seen with the endotoxin infusion model shown the preliminary results. We anticipate an extension of the four-hour window of treatment after infection using seen with TREM1 LP17 with the TLT-1 peptide and in the combinational treatment. We expect that TLT-1 will increase the window of treatment by two to four hours compared to TREM 1 treatments, because reagents' whose primary mode of action is targeted for TLT-1 is targeted to effect at the later stages of sepsis. We expect to see the levels of TNF- α , IL-1 β , IL-6, and soluble TREM-1 show no correlations with survival during treatment with only TLT-1 LP17, but mimic published values for TREM-1 LP17 when the combinational treatment is used. We expect to see a decreased level of TAT complexes, TLT-1 soluble fragment, and D-dimers when using the TLT-1 peptide or scFv.

Foreseen Pitfalls/Alternatives: The combinational treatments may show no improvement over either one individually suggesting that these are targeting the same site. In this instance we will make peptides to other regions of TLT-1, test their effectiveness in these described sepsis models, and if they show efficacy, we will enroll them in similar synergy trails as described above.

Aim 2: Demonstrate TLT-1 can be used as a marker for the development of DIC, ARDS, and Sepsis: We will (i) develop an ELISA and dot blot assay to identify the TLT-1 extracellular domain in the blood; (ii) identify human cohorts suffering from various stages of sepsis, including DIC and ARDS, and evaluate them for the presence of TLT-1 in the plasma using dot blot and ELISA.

(i) Develop an ELISA and dot blot assay to identify the TLT-1 extracellular domain in the blood: ELISAs will be completed as reported above with the scFv. Levels of sTLT-1 in samples of plasma will be measured with the use of an immunoblot technique similar to that used for TREM 1. Briefly, we will use a monoclonal murine IgG1, or a goat polyclonal antibody directed against human TLT-1 (R&D Systems), 100 μ l of patient plasma will be dotted onto

a nitrocellulose membrane, dried, and coated with PBS supplemented with 5% milk. The nitrocellulose sheet will then be incubated for 60 minutes in the presence of antibody. After thorough rinsing, the sheet will be incubated for another 60 minutes with secondary antibody and washed in phosphate-buffered saline supplemented with 20 percent dimethylsulfoxide and incubated for 30 minutes with horseradish peroxidase–conjugated streptavidin (Bio-Rad). The enzyme substrate chromogen Opti-4CN (Bio-Rad) will be added. Each sheet will contain calibration samples of a known concentration of TLT-1. Colorimetric determination will be achieved by means of a Versadoc scanner and PDQuest image analysis software (Bio-Rad).

(ii) Identify human cohorts suffering from various stages of sepsis, including DIC and ARDS, and evaluate for the presence of TLT-1 in the blood using dot blot and ELISA: At admission to the intensive care unit, we will record the following items for each patient: age; sex; severity of the underlying medical condition, stratified according to the criteria of APACHE II score¹⁵; Sepsis-related Organ Failure Assessment score¹⁶ (range, 0 to 24; scores for each organ system [respiration, coagulation, liver, cardiovascular, central nervous system, and kidney] ranged from 0 [normal] to 4 [most abnormal]); reason for admission to the intensive care unit; principal diagnosis; vital signs; respiratory variables; and results of routine blood tests and microbiological culture results. Survival or death in the intensive care unit will be assessed during a follow-up period up to 28 days. The attending physician will prescribe microbiological tests and antimicrobial therapy according to the usual practice of the intensive care unit, without interference by the research team. Two intensivists, retrospectively, will review all medical records pertaining to each patient and independently classify the diagnosis as the systemic inflammatory response syndrome (that is, no infection, sepsis, severe sepsis, or septic shock) at the time of admission, according to established consensus definitions. Intensivists will be blinded to results. We will compare TLT-1 values to two other markers used to measure the probability of sepsis procalcitonin and soluble TREM-1. Samples will be gathered within 12 hours after admission and study enrollment, 5 mL of whole heparinized blood will be drawn through an arterial line for measurement of procalcitonin and soluble TREM-1 levels. Plasma will be collected by centrifugation at 4° C, separated into aliquots, and stored at -80° C until the day of assay. Plasma procalcitonin concentrations will be measured by using an immunoassay with a sandwich technique and a chemiluminescent detection system, according to the manufacturer's protocol (LUMITest). Plasma soluble TREM-1 levels will be assessed as described elsewhere¹³

Expected results: We expect to see a positive correlation of the presence of the soluble fragment and the progression of sepsis and, as patients recover, we expect to see a decrease in the amount of soluble TLT-1. During the course of these experiments, we estimate to evaluate approximately 150 patients in various stages of sepsis over the next year. We expect to be able to correlate the presence of TLT-1 in the plasma with each stage of sepsis, including differences associated with DIC and ARDS, allowing the presence of TLT-1 to be used as a diagnostic tool for these conditions

Foreseen Pitfalls/Alternatives: This is a fairly straightforward aim. We have experience on all procedures listed. The most likely problem here is the ability to detect low enough levels of TLT-1 to properly correlate TLT-1 to the stages of sepsis. In this case we will use captured ELISA to increase sensitivity of our assay.

Statistical Analyses: Data will be entered, and submitted to posterior verification and summary frequencies before analysis, to identify missing values, non-concordant values or any discrepancy in the data. This will allow us to determine the correct amount of subjects to use per experiment and the best statistical test, considering the results of the normality test. Changes or alteration in aggregation will be evaluated by using Two-group Comparative Model (e.g. Chi-square and Student *t*-test). Analysis of variance (ANOVA) will be used to compare more than two groups. We will use rates of proportions to determine the amount of mice to use per group for the cecal ligation and endotoxin infusion experiments¹⁷. In all other experiments group size was determined by experience and published results from similar works. Serum sTREM-1 and

cytokines levels will be expressed as mean (+/-SD). The protection against LPS lethality by LP17 will be assessed by comparison of survival curves using the Log-Rank test. All statistical analyses will be completed using Statview software (Abacus Concepts) or Prism (GraphPad) and a two-tailed t-test. P0.05 will be considered significant. The statistical analyses will be performed at the UCC biostatistics core facility.

Future Directions: We are excited about the possibilities that this work presents. We have already positioned ourselves to take the next steps to move this work forward. The steps include the molecular modeling of the TLT-1 extracellular domain to identify the residues important for these interactions described above. Once we have identified these residues and confirmed them by mutational analysis, we have agreements with our collaborators to crystallize the TLT-1 active site. This will further facilitate the design of light and easily storable therapeutics that can be used more effectively for treatments in the hospital or the battlefield.

Relationship of this work to current research in your lab: Our institution has recently received an infrastructure grant to support an expansion of its Anatomy Department to include Cell Biology. The grant supported the creation of lab space, and the provision of basic supplies for two new faculty positions; one of which I was recruited to fill. I was charged with creating an externally-funded research program that would train graduate students in the biomedical sciences.

We were the first to describe TLT-1. As demonstrated in our preliminary results, we have developed reagents that specifically recognize the extracellular and intracellular domains of TLT-1. The laboratory I am developing focuses on platelets and their role in CVD, sepsis, as well as using TLT-1 to understand the mechanism of α -granule secretion. Our discovery and initial characterization of the TLT-1 and its potential role in thrombosis and hemostasis places our laboratory in a unique position to examine this interaction in detail.

G. Facilities Available

Laboratory space of 1000 sq. ft is available to the Principal Investigator. This space is equipped with tissue culture room, fume hoods, a shielded area for radiation use, a general laboratory bench area and a platelet aggregometer measuring platelet aggregation. The PIs have access to work space in the Department of Microbiology and Immunology. We have a proteomics core at the Universidad Central del Caribe. Our common facilities room is equipped with a Bio-Rad VersaDoc 3000 multi-imager for visualizing non-isotopic fluorescent, chromogenic and chemiluminescent-labeled samples. The VersaDoc has UV and white light transmission illumination for image capturing and analysis of blots, film and opaque samples. PDQuest image analysis software is used for gel image analysis. SA, flow cytometry, real-time polymerase chain reaction, FISH, and a microarray reader. We have recently purchased a FV1000 Olympus three channel confocal microscope with expected delivery in January 2007.

The university hospital, Dr. Ramon Ruiz Arnau, located in Bayamon, Puerto Rico, is a university and district hospital inaugurated in 1978. It is a tertiary hospital with 500 beds distributed in 5 major areas: Internal Medicine, Pediatrics, OB-GYN, Surgery and E.R. Also located within the Internal Medicine floor is an Intensive Care Unit and a Coronary Care Unit with continuous electronic monitoring of patients. Also, within the Pediatric ward, is a Pediatric Intensive Care Unit and Neonatal Intensive Care Unit. The Surgery department is composed of 8 large operating rooms and a ward. The Emergency Room is a modern and ample area, divided by specialties. Within ER there is a huge trauma room and cardio room for critical patients. The medical laboratory is very large, with modern equipment to process samples. This laboratory also includes a pathology lab with autopsy room.

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- (15) Knaus WA, Draper EA, Wagner DP, Zimmerman JE. APACHE II: a severity of disease classification system. *Crit Care Med* 1985 October;13(10):818-29.
- (16) Vincent JL, Moreno R, Takala J, Willatts S, De MA, Bruining H, Reinhart CK, Suter PM, Thijs LG. The SOFA (Sepsis-related Organ Failure Assessment) score to describe organ dysfunction/failure. On behalf of the Working Group on Sepsis-Related Problems of the European Society of Intensive Care Medicine. *Intensive Care Med* 1996 July;22(7):707-10.
- (17) Dell RB, Holleran S, Ramakrishnan R. Sample size determination. *ILAR J* 2002;43(4):207-13.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME Anthony Valance Washington		POSITION TITLE Assistant Professor	
eRA COMMONS USER NAME ANTWAS			
EDUCATION/TRAINING <i>(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)</i>			
INSTITUTION AND LOCATION	DEGREE <i>(if applicable)</i>	YEAR(s)	FIELD OF STUDY
Elizabeth City State University, Elizabeth City, NC	BS	May 1986	Biological Sciences
Southern Methodist University, Dallas, TX	Ph, D.	May 1998	Biological Science
Postdoctoral Fellow, Emory University School of Medicine, Atlanta, GA		1998 - 1999	Immunology
Postdoctoral Fellow, NCI, Frederick, MD		2001 - 2005	Leukocyte signaling

A. Positions and Honors.**Positions and Employment**

- 2005 – Present Assistant Professor – Universidad Central del Caribe, Bayamón Puerto Rico – Studies of platelet function
- 2001 - 2005 Cancer Research Training Fellow – National Cancer Institute, Laboratory of Experimental Immunology, Cell Signaling Section
- 1999 – 2001 Volunteer – United States Peace Corps, Mongop High School, Papua New Guinea – Science Teacher
- 1998 – 1999 Fellowship – Emory University, Department of Gynecology and Obstetrics Emory University School of Medicine, Atlanta Georgia
- 1993 - 1993 Summer Internship - NIH fellowship, National Institutes of Health, Bethesda Maryland. Advisor - Dr. Polly Matzinger
- Summers Summer Teaching Assistantship ,- Bermuda Biological station for research., 1995 & 96 Bermuda.
- 1994 - 1994 Summer Internship - Exxon Corporation scholarship,- Bermuda Biological station for research., Bermuda.
- 1997 – 1997 Residence Life Coordinator - The Institute for Shipboard Education, Pittsburgh, PA.
- 1996 – 1997 Research Assistant -. Southern Methodist University, Dallas, TX
- 1992 – 1996 Teaching Assistant - Southern Methodist University, Dallas, TX
- 1993 – 1995 Hall Director - Southern Methodist University, Dallas, TX
- 1992 – 1998 Graduate Student - Southern Methodist University, Dallas, TX
- 1986 – 1992 Sales Associate - Pall Corporation, Glenn Cove, NY. Chemical filtration specialist.

Honors

- 2005 Fellows Award for Research Excellence-National Institute of Health

- 2002- Pay increase for exemplary postdoctoral scientists.
1997 - Recipient of the Sigma Xi Outstanding Graduate Student in Biological Sciences award.

Other Experience and Professional Memberships

- 2005-Present International Society of Thrombosis and Haemeostasis
2003-2005 Society for Leukocyte Biology
2002-2005 Center For Cancer Research Fellow and Young Investigators committee
2002-2005 NIH Speakers Bureau
2001-2005 Felcom (NIH Fellows Committee) National Cancer Institute Basic Science
Representative: Job Fair Committee Chair (2004)

Supervision of Students from Underrepresented Groups in Research

- 2003 Robert Feltz –Mutational Analysis of the TLT-1 cytoplasmic domain.
2004 Andrea Allen – Identification of Protein-Protein interaction of the TLT-1 extracellular domain.
2005 Maia Chisholm – Identification of the TLT-1 extracellular domain in murine and human serum.

Cross Departmental Research Collaborations

- 2002 E. Cho – NCI Imaging Facility NCI, Frederick MD – Identification of TLT-1 in the α -granules of platelets
2003 P. Schwartzberg –Human Genome Institute, Bethesda MD – Targeted deletion of the TLT-1 gene
2003 J. Lubkowski – Macromolecular Structure group NCI, Frederick MD – Determination of the Crystal Structure of TLT-1.
2004 T. Mori – Molecular Targets Group NCI, Frederick MD – Identification of Single Chain Fragments Specific for TLT-1.

B. Selected peer-reviewed publication (in chronological order)

1. **Washington AV**, Quigley L, McVicar DW. Initial characterization of TREM-like transcript (TLT)-1: A putative inhibitory receptor within the TREM cluster. *Blood*. 2002 Nov 15;100(10):3822-4.
2. **Washington AV**, Rebecca L. Schubert, Laura Quigley, Theresa Disipio, Robert Feltz, Edward H. Cho² and Daniel W. McVicar, TREM family member, TLT-1, is found exclusively in the α -granules of megakaryocytes and platelets *Blood*. 2004, Aug 15;104(4):1042-1047.
3. **Washington AV**, McVicar DW Receptors involved in the regulation of murine NK cells Current Protocols Immunol. A.1R-A.1R.8, 2002
4. Gattis JL, **Washington AV**, Chisholm MM, Quigley L, Szyk A, McVicar DW, Lubkowski J. The structure of the extracellular domain of triggering receptor expressed on myeloid cells like transcript-1 and evidence for a naturally occurring soluble fragment. *J Biol Chem*. 2006 May 12;281(19):13396-403.
5. **A. Valance Washington**, Barbara Giomarelli, Maia M. Chisholm, Laura Quigley, James B. McMahon, Toshiyuki Mori, Daniel W. McVicar. Inhibition of thrombin-induced platelet aggregation using human single chain Fv antibodies specific for TREM-like transcript-1 (Submitted)
6. **A. Valance Washington**, Laura Quigley, Robert Feltz, Jun Cheng Amalia Dutra, Evgenia Pak, Pamela L. Schwartzberg, and Daniel W. McVicar. *In vivo* Characterization of TLT-1: Evidence for a role in sepsis. (*Manuscript in preparation*)



UNIVERSIDAD CENTRAL DEL CARIBE
Department of Cell Biology

Aniara Corporation
6560 Gove st.
Mason Ohio 45040

To Whom It May Concern:

This letter is to assure the members of the Aniara review board that Dr. Washington is a faculty member at the Universidad Central del Caribe. Dr. Washington assumed his duties effective January 2006 as part of the Anatomy and Cell Biology Department. Dr. Washington is currently and will be an Assistant Research Professor at the time of award. His position is slated at 95% research and 5% teaching and administrative duties to the university. His opportunity for advancement is commensurate to all other Assistant Professors of his rank and time in grade.

Dr. Washington was chosen from the pool of applicants based on his Curriculum Vita and a personal interview. It was felt that he was a promising candidate with the potential to be a successful independent researcher. He is also considered to be a potential asset to the university in his ability to complement the current research being conducted and in applying his skills and techniques to research being conducted in cellular immunology, such as flow cytometry and confocal imaging. Accordingly, he has volunteered to supervise the confocal facility being inaugurated at UCC this fall. In his free time, Dr. Washington studies the Spanish language to enhance his cultural and professional acclimation in Puerto Rico.

Dr. Washington is part of a new research initiative supported by a NIH Research Centers in Minority Institutions program grant. The RCMI grant provided for the renovation of his laboratory space to include 800 square feet of laboratory space, an office, computer, and equipment designed for the study of platelet function. He also has complete access to our shared facilities, including a Versadoc imaging system, and the calcium imaging facilities.

Dr. Washington reports directly to me, Dr. Suranganie Dharmawardhane, the Cell Biology Department chief. He has received one year of protected time to organize his laboratory and to submit grants to various organizations. He is confident and persistent in conducting his duties, and we anticipate that he will achieve success in his endeavors.

Sincerely yours,

Suranganie Dharmawardhane, PhD
Chairman of the Department of Cell Biology