Innate Immunity in Chagas Heart Disease: The Role of Cathelicidin

A thesis submitted by

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Abstract

Cardiac stem/progenitor cells (CPCs) can repair the injured heart by replacing dead cells and/or secreting paracrine factors that trigger cell survival. Whether CPCs also work to prevent myocardial injury is less appreciated. We observed that CPCs, unlike cardiac myocytes and fibroblasts, are resistant to infection by the obligate intracellular parasite *Trypanosoma cruzi*, which causes Chagas disease. We determined that the observed resistance is due to a soluble factor secreted by CPCs because CPC conditioned media prevents *T. cruzi* from invading otherwise permissive cardiac cells. RNA-Seq, real-time PCR and ELISA revealed that mouse CPCs constitutively secrete cathelicidin-related antimicrobial peptide (CRAMP), and indicated that this CPC-secreted peptide is one of the factors which inhibits *T. cruzi* invasion of cardiac cells. Indeed, CRAMP antibodies specifically eliminate the infection-inhibitory activity of CPC conditioned media, allowing for *T. cruzi* to invade the otherwise protected cardiac cells despite being exposed to the conditioned media. Furthermore, synthetic CRAMP peptide and human counterpart cathelicidin LL-37, but not the scrambled control peptide, potently inhibit *T. cruzi* invasion of permissive cardiac cells. Cathelicidins are known for their broad range of antibacterial and antifungal activities, and CPCs likely utilize this antimicrobial agent for immune surveillance of other pathogens besides *T. cruzi*. Thus, our results suggest that CPCs preemptively act against protozoan pathogens by constitutively secreting the antimicrobial peptide cathelicidin, highlighting a novel, stem cell-mediated, innate host defense mechanism against microbial invaders of the heart.

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AMP	Antimicrobial peptide
AP	Alkaline phosphatase
CCC	Chronic Chagas cardiomyopathy
cDNA	Complementary DNA
CF	Cardiac fibroblast
CM	Cardiomyocyte
CoM	Conditioned media
CPC	Cardiac progenitor cell
CRAMP	Cathelicidin-related antimicrobial peptide
DMEM	Dulbecco's modified eagle's medium
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
PAMP/DAMP	Pathogen/danger-associated molecular pattern
qPCR	Quantitative polymerase chain reaction
RT	Room temperature
sCRAMP	Scrambled cathelicidin-related antimicrobial peptide
Trk	Tropomyosin-related receptor kinase

Chapter 1. Introduction

1.1 The Heart and Cardiac Progenitor Cells

The mammalian heart is dedicated to pumping blood throughout the body, and is central in driving vital bodily processes and delivering essential nutrients via the circulation. It is critical for the heart to be protected from permanent damage. This protection is undoubtedly crucial during development since fetal and neonatal hearts demonstrate a strong capacity to regenerate damaged tissue[1]. The regenerative events underlying restoration of heart tissue are driven by the repair or replacement of degenerated cardiomyocytes. After birth, cardiomyocytes have reduced capacity to proliferate which is correlated with the restricted capacity for the adult heart to undergo regenerative processes. This is apparent under certain pathological conditions that manifest in the heart. Heart pathologies such as myocardial infarction and cardiomyopathy can occur during adult life which involves drastic loss of cardiomyocytes thus leading to decreased functionality of the heart. The death of cardiomyocytes can be repaired through a process related to wound healing but ultimately leads to fibrosis. With these pathologies, certain inflammatory processes may greatly overwhelm the regenerative responses in the heart and can lead to scar tissue formation. These mechanisms of pathology are what has historically established the idea that the adult heart is a post-mitotic organ, and is not capable of post-natal regeneration. However, new insights arise in the field of cardiac and circulation research it is becoming more widely accepted that challenge these longstanding concepts. Previously unappreciated cardiac cell interactions are at play within the adult heart, and are vital in maintaining physiological heart tissue maintenance and repair. The heart is composed of a

variety of cell types, specifically: 1) myocytes, 2) fibroblasts, 3) endothelial cells, 4) smooth muscle cells, 5) autonomous peripheral nervous system (sympathetic and parasympathetic), and 6) resident cardiac progenitor/ stem cells (CPCs)[6-9]. The heart parenchyma is made up of cardiomyocytes, which constitute 25- 35% of cardiac cells and perform the most function of the heart, pump blood through the circulation, fibroblasts, which may be found at varying degrees of abundance in cardiac tissue[10], are largely responsible for the makeup of extracellular matrix (though they also play important role in activating cardiomyocytes), and CPCs, endowed with cardiac repair function, representing about 2% of cardiac cells[11]. Endothelial and smooth muscle cells are important in structure and function of cardiac blood vessels. The discovery of endogenous cardiac stem/ progenitor cells (CPCs) has revealed a novel niche from which adult stem cells can be found. It has been observed that these cells proliferate in patient hearts after myocardial injury[13], and demonstrate differentiation capability to the cardiomyocyte lineage[14]. These findings have stimulated great interest in the cardiology field into understanding the mechanisms of cardiac pathologies since accurate modeling of the various situations that cause disease in patients has not yet been fully developed. Specific mechanisms of onset of disease remain to be discovered for autoimmune and pathogen dependent pathophysiology. To add another layer of complexity, there are still many questions to be answered regarding the physiological functions that CPCs play in protecting the heart from lasting injury.

1.2 Chagas' Disease

Chagas disease is a neglected tropical illness that affects millions of people in the Americas, Western Europe and Australia[2]. The disease is caused by the protozoan

flagellate *Trypanosoma cruzi*, which survives and replicates intracellularly in the cytosol of a variety of host cells in various tissues. Blood-sucking triatomine insects transmit the disease during a blood meal, as *T. cruzi*, from the insect feces, gains access to cells in the skin through abrasion or in mucosal surfaces such as in the conjunctiva. In addition, *T. cruzi* infects humans via blood transfusion, organ transplantation, congenitally via the placenta, and by ingestion of contaminated food or drink[2].

The disease progresses through two phases: acute and chronic. The acute phase typically begins within 4- 8 weeks after the initial infection, and lasts 3- 4 months[2]. The majority of acute phase cases progress largely unnoticed because clinical symptoms are non-specific such as fever, subcutaneous edema and malaise[3]. However, *T. cruzi* can cause acute myocarditis and meningoencephalitis that develop in children and in some adults[4]. *T. cruzi* spreads throughout the body via the circulation (parasitemia), and infects most organs like the heart, liver, and central nervous system. Following the acute phase, innate and adaptive immune responses significantly reduce the parasitic burden, and the host enters the chronic indeterminate phase, in which patients do not exhibit symptoms or pathology, and this can last a lifetime in approximately 60% infected individuals, whereas 15- 30% develop life-threatening cardiomyopathy (chronic Chagasic cardiomyopathy, CCC) years or decades after the initial infection[2]. Very low and often undetectable levels of parasites in the blood and other tissues, whether indeterminate or symptomatic, characterize chronic infection.

The heart is therefore the most important organ in Chagas disease pathogenesis, as CCC inevitably progresses to death. Despite its importance, the interaction of *T. cruzi* with the heart is poorly understood. It stands to reason that a better understanding of the mechanisms

governing *T. cruzi* infection of the heart will provide novel mechanistic insights and potentially give clues to the discovery of innovative regenerative therapeutics.

1.3 Antimicrobial Peptides

The innate immune system protects the host from a broad range of pathogens as a first line of defense. It is composed by a variety of immune cells which can sense pathogen and danger-associated molecular patterns (PAMPs and DAMPs respectively) via recognition of certain evolutionarily conserved molecular profiles. However, before the cells of the innate immune system mobilize to respond to potential invading pathogens, various host defense molecules are constitutively distributed across multiple organs and mucosal barriers to provide immediate blockades against various pathogens. Some of the most important molecules of this innate host defense system are the diverse repertoire of antimicrobial peptides (AMPs) which include lysozymes, defensins, histatins, RNases, various chemokines, and cathelicidins. These AMPs elicit broad antimicrobial activities against bacteria, fungi and viruses while also having chemotactic effects on cells of the innate and adaptive immune system. The abilities of AMPs to kill certain pathogens stems mainly from their ability to interact with certain structures on the surface of cells. Typically, AMPs have been known to exhibit a net positive charge and can readily interact with negatively charged molecular structures on pathogen cell surfaces[30, 31].

1.4 Cathelicidin

Cathelicidin is produced as a precursor with a conserved N- terminal cathelin- like domain, and subsequently matured by extracellular proteases in which the antimicrobial C- terminal domain becomes active[15]. The mature form of the peptide is alpha helical, amphipathic and

cationic which enables it to interact readily with negatively charged cellular biomolecules. These attributes contribute to various mechanisms by which cathelicidin is able to block pathogen survival in a given host. These mechanisms include membrane pore formation, interference of microbial environment and membrane component biosynthesis, inhibition of cytokinesis by complexing with DNA, and interactions with biofilms which can lead to a reduction of bacterial cell attachment[16]. In human and mice, cathelicidin can be found at very high concentrations in the skin, but is also synthesized in great quantities by macrophages and activated neutrophils, and associates with released neutrophil extracellular traps (NETs) where it exhibits its antimicrobial activities in conjunction with cellular innate immune responses[17]. Cathelicidin is able to defend against various bacterial and fungal pathogens such as *Acinetobacter baumannii*[15], *Bacillus subtilis*, *E. coli*[18] and *Candida albicans*[19]. Besides having antimicrobial effects against pathogens, cathelicidin demonstrates wound healing properties[20], enhances stem cell paracrine functions[21, 22] and regulates inflammatory events outside of the mucosal tissues[23]. Whereas the aforementioned mechanisms of cathelicidin have been extensively studied for bacterial and fungal pathogens, the role that cathelicidin plays during *T. cruzi* interactions with mammalian host cells is completely unknown. The only investigations regarding AMP response to *T. cruzi* have been demonstrated in the parasite insect vector, *Rhodnius prolixus*, in that AMPs in the midgut affected the insect microbiota[24]. The other few published works on *T. cruzi* and AMPs refer to AMPs from animals than are non-*T. cruzi* hosts such as mellitin that is made by bees[27].

1.5 Rationale for the Project

We have discovered that CPCs are resistant to invasion by *T. cruzi*, which raised the possibility that CPCs provide a certain innate immune function in their niche within the heart tissue. Through an initial gene expression screen using RNA-seq, and confirmed by qPCR and ELISA, we found that CPCs synthesize cathelicidin-related antimicrobial peptide (CRAMP). This prompted us to investigate the role that CPC-derived CRAMP played in *T. cruzi* invasion dynamics by using *in vitro* infection models for Chagas disease. For the first time, we demonstrate that CPCs, a novel source of stem cells found in the adult heart, make CRAMP which is antimicrobial against pathogenic invaders thus protecting other heart cells from harm.

Chapter 2. Materials and Methods

Parasites. Experiments were performed with the *T. cruzi* Colombian strain propagated in Vero cells. Infective trypomastigotes were harvested from supernatants by initial low-speed centrifugation (500 x g, 5 min) to remove host cells and debris, followed by high-speed centrifugation (1000 x g, 15 min) to pellet the parasites, which were resuspended in 1% FBS in Dulbecco modified Eagle medium (DMEM).

Mice. Female C57BL/6 and breeding pairs of B6.129X1-*Camp*^{tm1Rlg}/J mice (age, 6 to 8 weeks) were from The Jackson Laboratory (Bar Harbor, ME). All mouse experiments were approved by the Institutional Animal Care and Use Committee (IACUC) and the Division of Laboratory Animal Medicine (DLAM) of the Tufts University School of Medicine.

Cell lines and primary cell cultures

(i) **Cell lines.** H9C2 cells (ATCC CRL-1446) (rat cardiomyocytes "CMs") were maintained in 10% FBS-DMEM, and Vero cells (used to propagate *T. cruzi*) were maintained in 1% FBS-DMEM. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO2.

(ii) **Primary cultures.** Sca1^{+} cardiac progenitor cells (CPCs) were isolated from C57BL/6 female mice using the Cardiac Progenitor Isolation Kit (Sca-1) mouse (Miltenyi Biotec). Mouse hearts were incubated with 600 U/mL of Collagenase II solution in HBSS (Worthington Biochemical #CLS-2) with 60 U/mL DNase (Roche #10 104 159 001), and disassociated in gentleMACS C tubes (MACS #130-093-237, #130-096-334) using a gentleMACS Dissociator (MACS #130-093-235) following manufacturer's instructions for all subsequent steps throughout the procedure. The isolated CPCs were cultured in 68.8% DMEM,

8.1% IMDM, 16.2% Hams F12, 5.9% FBS, 0.5% B27 Supplement, 0.5% GlutaMax, 0.25% Pen/Strep, 0.125% NEAA base containing 0.75 mM sodium pyruvate, 20 ng/mL basic fibroblast growth factor (bFGF), 6.25 ng/mL epidermal growth factor (EGF), 125 ng/mL thrombin, 1 ng/mL cardiotrophin-1, 25 μM β-mercaptoethanol and 0.25 μg/mL insulin. Primary cardiac fibroblasts (CFs) were isolated as previously described (Salvador et al, 2014), and were cultured in 34.75% DMEM, 34.75% Ham's F12, 20% FBS, 0.3% Pen/Strep, 0.4 mM L- glutamine, 1% NEAA base containing 3 mM sodium pyruvate and 1 μg/mL insulin.

Conditioned Media. CPCs were plated in 6 well culture plates at a seeding density of 5 x 10^4 cells per well in complete growth media, and allowed to attach overnight. CPC conditioned media (CoM) was then de- cellularized by centrifugation at 1000 x g for 10 minutes followed by filtration via a 0.22 μm filter. De- cellularized CoM was used the day of infection for incubation with parasites, or frozen at -80°C in aliquots for future molecular characterization experiments.

Peptides. Peptides were purchased from ABI Scientific, Inc. (Sterling, VA, USA). The sequences are given below:

CRAMP: GLLRKGGEKIGEKLKKIGQKIKNFFQKLVPQPEQ

LL-37 (Cathelicidin): LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES

sCRAMP: KIGIEQLKGIKGIPEKRPGKRFKIVGEFSNQKALQKLQL

qPCR. RNA was isolated from TRIzol lysates of cell monolayers or tissue samples that had been snap-frozen in liquid nitrogen and dissociated by a Tissue-Tearor mechanical

homogenizer (Biospec Products, Inc.). cDNA was synthesized using a Quantitect reverse transcription kit (Qiagen) according to the manufacturer's instructions. CRAMP mRNA levels were normalized to those of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using SYBR green (Qiagen). The following specific primers were used: CRAMP (forward primer, CTGTGGCGGTCACTATCACT; reverse primer, TCGGAACCTCACAGACTTGG) and GAPDH (forward primer, TGTCCGTCGTGGATCTGAC; reverse primer, CCTGCTTCACCACCTTCTTG).

Immunodepletion and Neutralization of CRAMP from CoM. De- cellularized CoM was subject to incubation with anti- CRAMP antibody (sc-34169; Santa Cruz Biotechnology, INC.) or normal mouse IgG_{2a} isotype control (sc-3979; Santa Cruz Biotechnology, INC.) at 2 µg/mL for 1 hour at room temperature and then 4°C overnight (>12 hours).

(i) Immunodepletion. Anti- CRAMP and isotype control incubated CoM was applied to a lab scale solid glass bead (11312A; Thermo Scientific Co, Pittsburgh, PA, USA)/ Bio- Gel® P-100 mesh (1504174; BioRad) column stacked with protein A sepharose™ (Amersham Biosciences AB, Uppsia Sweden) which was thoroughly equilibrated with 1X PBS prior to immunodepletion. Samples were re- applied through the column several times after the initial collection to ensure efficient antibody binding to the column. CoM and non- conditioned media (non- CoM) was also applied through the column in the same scheme for controls. The flow through was then sterile filtered via a 0.22 µm filter for use in parasite infection assays. The efficiency of the depletion was measured by ELISA, and CRAMP-depleted CoM was verified for deficient CRAMP concentration before use in infection assays.

(ii) Neutralization. The same methodology was used for neutralization experiments, however antibody incubated media samples were not applied to the protein-A/ sepharose column.

Competitive ELISA. CRAMP was incubated at 2 µg/mL in borate buffer on maxisorp high-affinity ELISA plates overnight. Anti- CRAMP antibody was incubated with CRAMP standards prepared in borate buffer (0-2 µg/mL) or cell- free conditioned media overnight. The ELISA plate was blocked (1% BSA in 1X borate buffer/ 0.1% sodium azide, 2 h at RT) and subsequently washed (1X borate buffer/ 0.1% tween) before incubating samples (2 h at 37°C). Wells were washed thoroughly, and incubated with secondary antibody (1 h at 37°C) (A4187: anti-goat IgG, alkaline phosphatase conjugated- Sigma), washed, and then incubated with detection solution (4-nitrophenyl phosphate disodium salt hexahydrate tablet dissolved in 1M glycine, 0.5M ZnCl, 0.5M MgCl). Absorbance readings were taken at A405 using a multi-plate reader. The concentration of CRAMP in conditioned media was determined by comparing the inhibition of binding of the sample with the CRAMP standards.

Antitrypanosomal Activity of Cathelicidin.

(i) Infection Assays. Readout cells (CMs or CFs) were plated at least 12 hours before infection in 96 well cell culture plates coated with 100 µg/mL (100µL) poly L-lysine. Trypomastigote parasites were collected as described, and diluted in 1% FBS-DMEM at a defined multiplicity of infection (MOI) containing various concentrations of CRAMP, Cathelicidin or sCRAMP at multiple times of incubation. Then, the readout cell monolayers from overnight plating were washed in serum free (SF)-DMEM and the treated parasites were added to the cell

monolayers for 3 hours after brief centrifugation (500 x g, 5 min). At that time, parasites were washed off in SF-DMEM and 1% FBS-DMEM (for CMs) or 1:5 primary cardiac fibroblast culture media (for CFs) was added to readout cells for a 4 day incubation to allow for amastigotigenesis. Vehicle controls contained the equivalent volume of molecular grade distilled water in infection media as in peptide treated samples. The inhibition of infection percentage was represented as the difference between the infected readout cells in the experimental group compared to the vehicle treated control group.

(i) Motility. Parasites were incubated with CRAMP, Cathelicidin or sCRAMP at 1 µM for 30, 60 and 180 minutes and 5 µM for 2.5, 5, 10, 20, 40, 60, 360 and 1440 minutes (24 hours) in a 96 well cell culture plate. At each time point, the number of motile parasites was assessed by loading an aliquot of each sample condition in a hemocytometer. Motility was calculated as the number of motile parasites in 4 fields of the hemocytometer. Vehicle controls contained the equivalent volume of molecular grade distilled water in infection media as peptide treated samples.

(ii) Morphology. Parasites were incubated with CRAMP and Cathelicidin at 0.2, 1 and 5 µM for 3 hours at 37°C in a poly L-lysine coated 96 well plate. After 3 hours, parasites were centrifuged at 500 x g RPM for 5 minutes and incubated at 4°C for 30 minutes to allow the parasites to adhere to the bottom of each well. The plate was abruptly inverted to expel the supernatant, and then air dried in a laminar flow hood for 6 hours. sCRAMP and vehicle controls (Veh) were treated in the same scheme as experimental samples. After dehydration, samples were stained using PROTOCOL™ Hema 3™ solution II (122-952; Fisher Scientific Company LLC,

Kalamazoo, MI) following manufacturer's instruction. Samples were viewed using an Olympus IX70 light microscope, and images were captured using Spot Advanced software.

(iii) MTT Assay. Parasites were incubated with CRAMP, Cathelicidin or sCRAMP at 1, 5 or 50 µM (37°C, 3 h) in a 96 well cell culture plate. Following this incubation, parasites were incubated with MTT at 1 mg/mL (37°C, 4 h) solubilized in DMSO, and then read at 540 nm spectrophotometrically. Percent viability was calculated by dividing the absorbance reading of the experimental group by that of the vehicle treated control.

Data Representation and Statistical Analysis

All data was interpreted and represented using GraphPad Prism 5 software. Statistical analysis packages which were available in this software were used to define statistical significance. ANOVA was used as a comparison of variances between multiple groups, and then further analyzed using Tukey follow-up test to determine specific comparisons.

Chapter 3. Results

3.1 Cardiac progenitor cells resist T. cruzi invasion

T. cruzi readily invades cardiac myocytes (CM) and fibroblasts (CF) (as it does most other known nucleated mammalian cells that spread on a surface in culture). Under the conditions of the infection assay we use routinely in the lab, approximately 18% of cells are infected by *T. cruzi* at an MOI (multiplicity of infection) of 20 (**Fig 3.1A**). On the other hand, only about 0.5% CPCs are infected under the same conditions (**Fig 3.1A**). This experiment has been repeated many times by multiple members of the lab. Hence, one can conclude that, for whatever to-be-determined reason, either CPCs are resistant to *T. cruzi* invasion due to the scarcity of invasion receptors or some other mechanism critical for *T. cruzi* entry into host cells. Alternatively, *T. cruzi* cannot invade CPCs not because of an invasion defect mechanism inherent to the stem cells, but rather because CPCs turn *T. cruzi* non-invasive to the stem cells. If that were to be the case, then *T. cruzi* will be unable to invade known permissive cells after interacting with CPCs. The first explanation is predictable, but it is not exciting, whereas the second hypothesis is tantalizing because it is not obvious in parasitology. We chose to pursue the tantalizing explanation for the poor *T. cruzi* invasion of CPCs. If CPCs inactivate *T. cruzi* invasion, it may occur at the level of

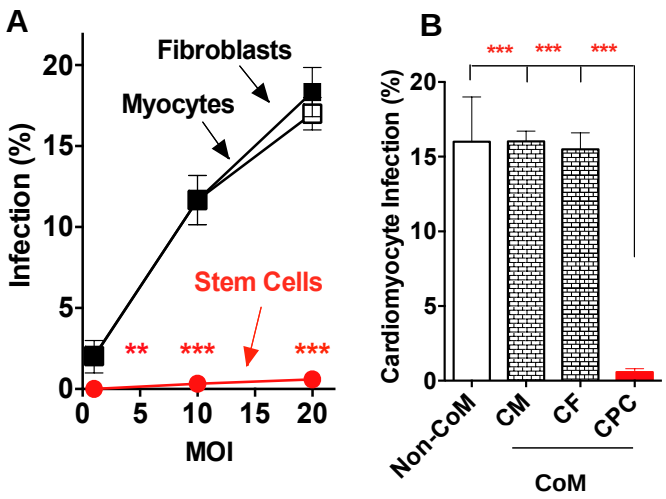

Fig 3.1 Cardiac progenitor/ stem cells are non-permissive to *T. cruzi*, and secrete agents that inhibit infection.
(**A**) Primary cardiac progenitor/ stem cells (CPCs) were isolated from adult C57BL/6 mice and fibroblasts from newborn mice. Myocyte is the cardiac cell line H9C2. Cells (in triplicate) were incubated with trypomastigotes (Colombian strain) at the indicated multiplicity of infection (MOI) for 3 h (to allow cell entry), washed to remove uninvaded parasites, and grown for 4 d for amastigotes to replicate, and stained with Diff-Quik by phase-contrast microscopy (to quantify infected cells containing ≥ two amastigotes/cell, in more than 400 cells). (**B**) Conditioned medium (CoM) collected after overnight cultures of cardiac myocytes (CM), fibroblasts (CF) and stem cells (CPC), and medium that had not been in contact with cells (Non-CoM) were incubated with *T. cruzi* (3 h, 37°C) and used to infect cardiomyocytes at MOI of 20. Infected cardiomyocytes were quantified by phase- contrast microscopy as in (**A**). ***, $p<0.001$.

cell-cell interaction, that is, at the time the parasite binds to CPCs during the first phase of entry. Otherwise, inactivation of invasion could result from the secretion of a protective factor by CPCs that turns *T. cruzi* non-invasive. We tested this concept of CPCs having the ability to cripple *T. cruzi* invasiveness because it is straightforward and easier to do experimentally. That is because this protective factor must be in CPC conditioned medium (CoM), whether induced by *T. cruzi* or not (in which case the production of such factor could be considered constitutive). Therefore, we incubated *T. cruzi* with CoM from CPCs, CMs or CFs, harvested after overnight culture. It is remarkable that CoM from CPCs, but not from CMs or CF, potently inhibited *T. cruzi* invasion of CMs (**Fig 3.1B**). These results were confirmed in multiple independent experiments. We therefore concluded that CPCs secrete some protective factor

constitutively. Whether CPCs augment expression of this protective factor in response to *T. cruzi* will be determined in the future.

3.2 CPCs Express the Antimicrobial Peptide Cathelicidin-Related Antimicrobial Peptide (CRAMP) constitutively

The idea that CPCs constitutively secrete this inhibitory factor raised the possibility that it is an antimicrobial peptide (AMP). AMPs are an important innate immune mechanism prevalent in the animal and plant kingdoms, widely studied in viral, bacterial and fungal infections[24-26]. Not much is known about the role AMPs play in protozoan infections. As noted earlier, a few AMPs have been studied in the context of *T. cruzi* infection but the AMPs studied (mellitin and prolixicin) are made by insects[27, 28]. Still, we decided to test the possibility. First, we checked for expression of AMPs in CPCs using RNA-Seq. We found that CPCs selectively express CRAMP (**Fig 3.2A**). We confirmed the RNA-seq finding by qPCR, which also revealed that CRAMP is expressed by CPCs and not CMs and CFs (**Fig 3.2B**). After discovering this, we sought to confirm that the actual CRAMP protein is in fact secreted into CPC conditioned media. This was accomplished by performing competitive ELISAs that were developed in-house, and then optimized to analyze the conditioned media (CoM) from CPCs, cardiomyocytes (CMs) and cardiac fibroblasts (CFs). As expected, CRAMP could be detected abundantly in CPC CoM but not in CoM from CMs or CFs (**Fig 3.2C**). This gave some strength to the main idea that CRAMP is an important factor in CPC CoM which mediates protection of cardiomyocytes and cardiac fibroblasts from infection by *T. cruzi.* After discovery of these findings, we

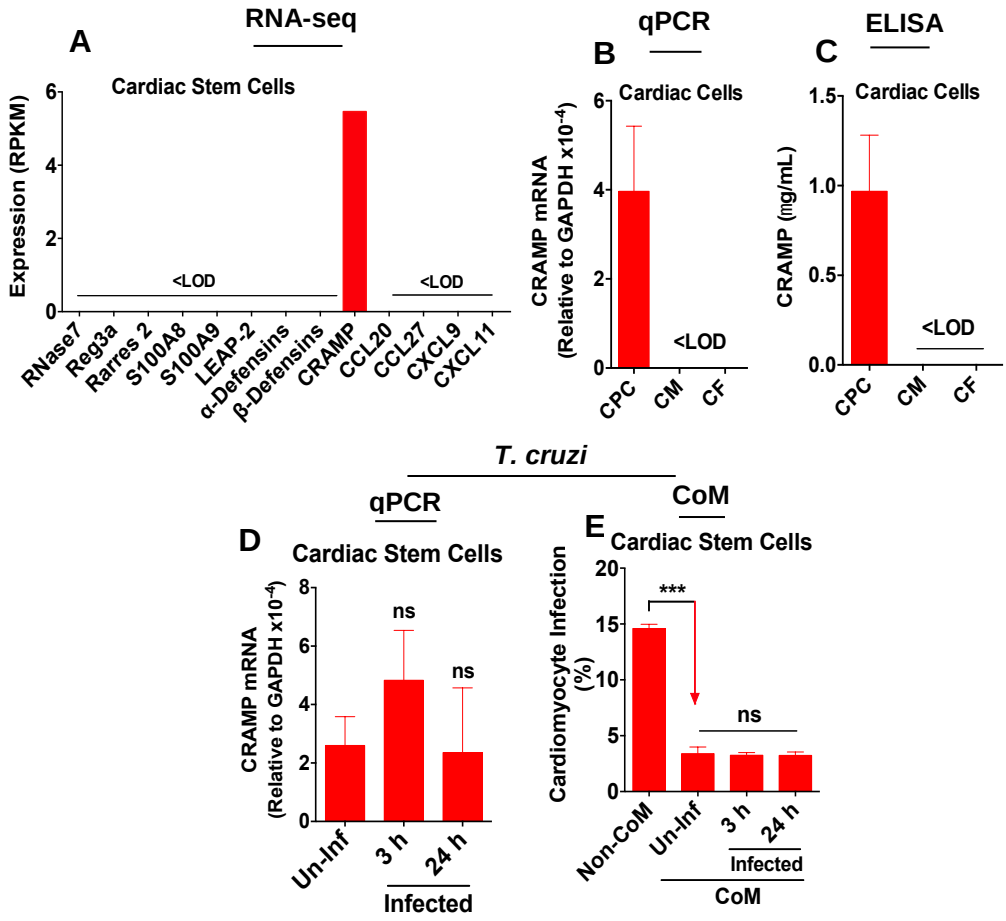

Figure 3.2 Selective and constitutive expression of CRAMP by cardiac stem cells. (**A, B, C**) *Constitutive Expression.* CRAMP is expressed by cardiac stem cells, and not by cardiac myocytes (CMs) and fibroblasts (CFs), as revealed by (**A**) RNA-seq, (**B**) qPCR, and (**C**) ELISA. (**D**) CRAMP mRNA levels in CPCs remain at basal levels after 3 and 24 hours of *T. cruzi* infection, and (**E**) this stimulation does not affect inhibitory action of CPC CoM. qPCR and ELISA measurements in triplicates; results have been reproduced in three experiments. The concentration of CRAMP secreted by cardiac stem cells is 0.9 µg/ml ± 0.1. Reg3a, regenerating family member 3 Alpha; Rarres 2, retinoic acid receptor responder 2; LEAP-2, liver enriched antimicrobial peptide 2; CCL20, C-C motif chemokine ligand 20; LOD, limit of detection. CRAMP mRNA was quantified in uninfected (Un-inf) cardiac stem cells. Conditioned supernatants of corresponding cells were assessed for their inhibition of *T. cruzi* infection. ***, $p<0.001$; ns, not significant.

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asked the questions of whether exposing *T. cruzi* to CPCs regulates expression of CRAMP.

This idea stemmed from the well-known observations that pathogens typically stimulate

expression of genes encoding antimicrobial agents[33]. Upon stimulation of CPCs with *T. cruzi*, we found that *T. cruzi* did not induce changes in CRAMP mRNA levels in CPCs after 3 and 24 hours of infection (**Fig 3.2D**). Furthermore, the CoM from the CPCs stimulated in this manner demonstrated no change in inhibitory action as seen in our infection assays (**Fig 3.2E**). These data revealed to us that CPCs express CRAMP specifically, as compared to other cells in the heart, and constitutively upon infection with *T. cruzi*.

3.3 Murine and human cathelicidin inhibits *T. cruzi* invasion of cardiomyocytes and cardiac fibroblasts in a dose- and time-dependent manner

The results displayed in **Fig 3.2** suggest that CRAMP, as well as the human counterpart cathelicidin, may contribute to the antimicrobial action of CPC CoM against *T. cruzi*. We therefore tested whether CRAMP and cathelicidin exhibit anti-infective activity for *T. cruzi* using cardiomyocytes (CMs) and cardiac fibroblasts (CFs) as hosts. *T. cruzi* was incubated with a wide range of concentrations (4 nM to 50 μM) of the synthetic peptides for 3 hours, added the mixtures to CMs for another 3 hours, and determined the degree of CM inhibition of infection 4 days later by phase-contrast microscopy of Diff-Quick-stained monolayers. Clearly, both CRAMP and cathelicidin, but not sCRAMP, inhibit *T. cruzi* invasion of CMs and CFs dose-dependently (**Fig 3.3A and B**). In fact, at the indicated concentrations of the AMPs, the results from **Fig 3.3A and B** were nearly identical between the two different cell types. In order to determine whether cathelicidin elicited antimicrobial activities on *T.*

cruzi in a time-dependent manner, *T. cruzi* were incubated with the AMPs at a concentration that inhibited approximately 50% of the infection (this was determined to be 1 μM by the IC_{50} curve in **Fig 3.3A** but for various amounts of time). We determined that the AMPs inhibited *T. cruzi* infection of cardiomyocytes in a time-dependent manner, in that by increasing the amount of time that AMPs were incubated with *T. cruzi* the less numbers of cardiomyocytes were infected (**Fig 3.3C**).

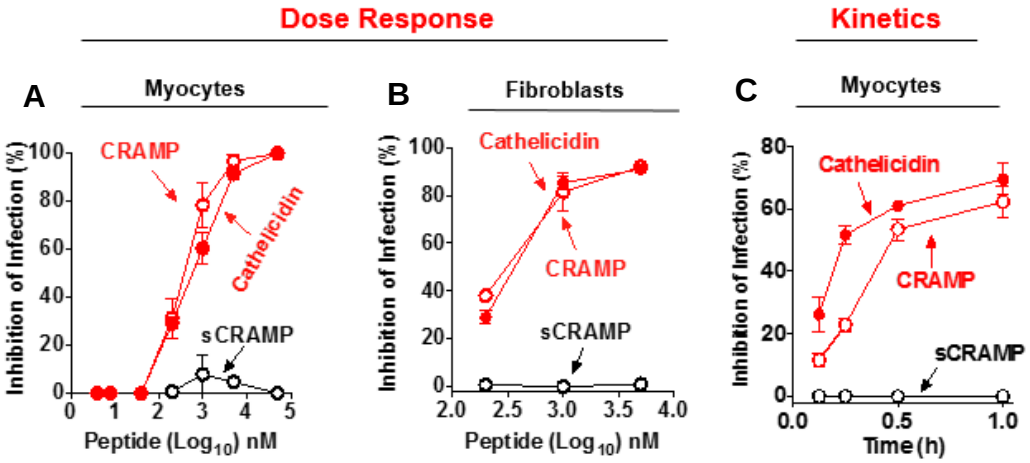

Figure 3.3 Cathelicidin inhibits *T. cruzi* invasion of cardiac fibroblasts and myocytes in a dose-, time- and parasite-dependent manner.
(**A**) Dose-response inhibition of *T. cruzi* invasion of cardiomyocytes by synthetic human cathelicidin and murine cathelicidin (CRAMP). sCRAMP is a scrambled version of CRAMP. *T. cruzi* was incubated with the synthetic peptides for 3 h and added to cardiomyocytes (H9C2) adherent to plastic substratum (96-well plates), washed to remove uninvaded parasites, and internalized parasites allowed to differentiate and replicate for 4 d. Infection assessed by phase-contrast microscopy. Graph represents the combination of four experiments. (**B**) Dose-response inhibition of *T. cruzi* invasion of cardiac fibroblasts by synthetic human cathelicidin and murine cathelicidin (CRAMP). sCRAMP is a scrambled version of CRAMP. (**C**) Time-course of cathelicidin inhibitory activity of cardiomyocyte invasion. *T. cruzi* was incubated with human and murine cathelicidins (1 μM) for 7.5, 15, 30 and 60 min, washed to remove unbound peptides, then used to infect (MOI 20) cardiomyocytes; cell infection was quantified after 4 d.

3.4 Cathelicidin is responsible for cardiac stem cell resistance to *T. cruzi* infection

To this point, our results indicate two novel findings: (1) CPCs express CRAMP, but CMs and CFs do not, and (2) murine and human forms of cathelicidin potently inhibit *T. cruzi* infection of CMs and CFs in a dose- and time-dependent manner. These data gave strong support to the idea that CPCs mediate protection of CMs from *T. cruzi* infection by secreting CRAMP. However, we had yet to prove this concept. Therefore, we developed immunodepletion and neutralization protocols to determine the effects of removing (immunodepletion) or blocking (neutralization) CRAMP in CPC CoM on *T. cruzi* infection of CMs. Miniature

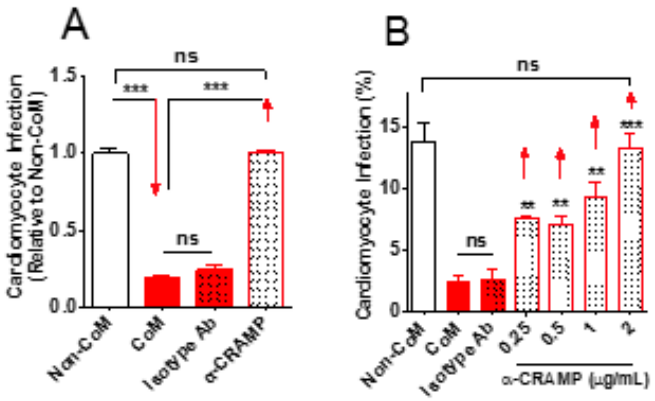

Figure 3.4 Murine cathelicidin (CRAMP) is responsible for cardiac stem cell resistance to *T. cruzi* infection.
(**A**) Cardiac stem cell conditioned media (CoM) was mixed with 2 μg/ml anti-CRAMP antibody (α-CRAMP) (37ºC for 30 min, and overnight at 4^0C) or isotype-matched control antibody (Isotype Ab), and the mixtures passed twice through protein-A Sepharose column to remove α-CRAMP/antibody immune complexes. The flow-through was assayed for inhibition of *T. cruzi* Colombian (MOI 20) infection of cardiomyocytes (in triplicate), in parallel with CoM (CoM, positive inhibitory control) or non-conditioned medium (Non-CoM) (negative inhibitory control) that did not go through protein A-Sepharose. Results are expressed relative to *T. cruzi* infection in Non-CoM, set at 1.0. This experiment was repeated three times, and each gave result similar to that displayed here. (**B**) CoM untreated with antibody (CoM) or treated with the indicated concentrations of neutralizing α-CRAMP antibody (60 min, 37ºC), or corresponding isotype-matched antibody (Isotype Ab, shown here at 2 μg/mL) were allowed to infect (MOI 20) cardiomyocytes under standard infection assay conditions. Similar results were obtained in two other experiments. **, $p<0.01$; ***, $p<0.001$.

protein-A columns were constructed using BioGel resin and materials readily available in the lab along with a defined amount of protein-A conjugated sepharose. CPC CoM was incubated overnight with anti-CRAMP antibody or isotype control antibody, and then applied to the protein-A column for immunodepletion. Alternatively, CoM reacted overnight with anti-CRAMP antibody at various concentrations and was termed "neutralized" without being applied to the protein-A column. After extensive immunodepletion and neutralization, the CoM was incubated with *T. cruzi* and then used to infect CMs using our well-established infection protocol. Immunodepletion of CRAMP from CPC CoM resulted in nearly the same result as in incubated *T. cruzi* with non-CoM (fresh media with no cell contact) (**Fig 3.4A**). However, isotype control antibody incubated CoM resulted in the same inhibitory potential as unmodified CoM (**Fig 3.4A**). Similarly, increasing the concentration of anti-CRAMP antibody used for neutralization in CPC CoM resulted in increased numbers of infected cardiomyocytes (**Fig 3.4B**). These results made the connection between our findings that CRAMP is expressed by CPC, and that CRAMP (and human cathelicidin) inhibits *T. cruzi* infection of CMs since CPC CoM inhibits *T. cruzi* infection but not CoM from CMs and CFs.

Chapter 4. Discussion

When *T. cruzi* infects the heart, it has the opportunity to penetrate and thrive in cardiac myocytes, fibroblasts, smooth muscle cells, endothelial cells, autonomous nervous system, and/ or resident cardiac stem/ progenitor cells (CPCs). *T. cruzi* interaction with cardiomyocytes is by far the most studied in the Chagas disease field, followed by cells of the endothelium, smooth muscle, and nervous system[11]. There are few papers related to invasion of fibroblasts[12-13, 29]. Furthermore, there are no published papers related to *T. cruzi* interaction with CPCs, despite the importance of CPCs in heart physiology and pathogenesis, and given their important role in tissue repair and maintenance[14, 15]. The overall scope of this work was to further investigate the host-pathogen interaction between the host and *T. cruzi* in the context of heart infection. More specifically, one of the main objectives from this work was to reveal some of the less appreciated interactions between CPCs and *T. cruzi.*

I started working in the Perrin lab in July 2015 while taking class in the MERGE-ID program. At the time, my adviser and lab members were stunned to discover that CPCs were resistant to invasion by *T. cruzi.* This is because *T. cruzi* in able to invade nearly every known mammalian cell, at least *in vitro*. If a cell attaches and spreads on a substratum such as plastic of a 96-well plate, *T. cruzi* will thrive inside the cells. In the case of the heart, as modeled by the given conditions of the discussed infection assay, *T. cruzi* invades approximately 15% of cardiomyocytes or fibroblasts, and only 0.5% of CPCs. That is, CPCs are 20-30-fold more resistant to invasion than cardiomyocytes and fibroblasts.

As one could imagine, there are many explanations for this unexpected inability of *T. cruzi* to invade CPCs. The most logical one is that invasion receptors *T. cruzi* uses to invade mammalian cells are lowly abundant in CPCs. Our lab has identified TrkA and TrkC as receptors for invasion in a variety of cells[17, 18] so a likely explanation for the non-permissiveness was that expression, either high or low, of TrkA, TrkC and other to-be-identified *T. cruzi* invasion receptors are different from other commonly studied host cells.

However, given I was a rotating student, I was assigned to explore the possibility that resistance of CPCs to invasion was due to the secretion of factors that would prevent *T. cruzi* from entering CPCs. If that were the case, then these factors would also inhibit *T. cruzi* invasion of widely studied cardiomyocytes and other bona fide host cells which are typically permissive to *T. cruzi* during infection. This would ultimately reveal a new concept in host cell invasion, not only by *T. cruzi* but also by other parasites such as T*oxoplasma*, *Cryptosporidium*, *Leishmania*, and so on, as we are not aware of such concept in parasitology. For this reason, I took on the project as a rotating student.

In short, the idea for my rotation had merit and was not unrealistic, after all. During my rotation, which extended from July to October 2015, we demonstrated that CPCs do indeed secrete a factor that blocks *T. cruzi* invasion of cardiac myocytes and fibroblasts that is present in CPC conditioned medium. As I joined the lab for my thesis, we thought to investigate the possibility that this protective factor is one or more of the known antimicrobial peptides (AMPs), that humans make more than 100 of them[18]. RNA-seq revealed that CPCs express five AMPs that are not expressed by cardiomyocytes, supporting our main hypothesis. We started by testing the AMP cathelicidin made by humans[19], which in mice is called cathelicidin-related antimicrobial peptide (CRAMP).

With this logic, we found that synthetic cathelicidin and CRAMP potently inhibit *T. cruzi* invasion of cardiac myocytes and fibroblasts.

Many AMPs were discovered for their antimicrobial activities and subsequently found to activate cells of the immune system, and vice versa for many other AMPs. Cathelicidin and CRAMP were discovered as AMPs[10] and subsequently as immune mediators[21, 22]. Interestingly, CRAMP has recently found to abort autoimmune reactions, specifically in development of type I diabetes[23]. Autoimmunity is thought to be a mechanism governing Chagas cardiomyopathy, raising the possibility of *T. cruzi*-cathelicidin recognition is involved in CCC development or lack thereof.

In sum, although AMPs are widely studied in the context of bacterial, fungal and viral infection[24-26], very little is known about their role in parasitic infections in general and *T. cruzi* invasion in particular. The few published works on *T. cruzi* and AMPs refer to AMPs from animals than are non-*T. cruzi* hosts such as mellitin that is made by bees[27, 32]. There are various mechanisms of action by which the diverse array of AMPs elicit their antimicrobial activities on these various pathogens. Cathelicidin elicits its antimicrobial activities on a wide range of pathogens by forming pores in the cell membrane, disrupting self-assembly of microbial structures such as biofilms and by binding DNA which halts cytokinesis in bacteria and fungi. After discovering that CRAMP and cathelicidin both potently inhibit *T. cruzi* from invading host cells, we were very interested in whether these AMPs do so via the mechanisms of action previously mentioned. Some preliminary results which we began to observe suggest that cathelicidin does not form pores in *T. cruzi* membranes as measured by fluorescent microscopy for PI staining, and does not affect their viability as measured by MTT (data not shown). Also, we observe that certain

concentrations of the AMPs inhibit *T. cruzi* motility but others do not while all concentrations still block *T. cruzi* from invading the host cells (to be confirmed by future experiments). These findings were quite fascinating in that these AMPs may be acting via a unique mechanism of action to inhibit *T. cruzi* invasion of host cells, and future projects would be carried out to expand upon these preliminary observations.

In conclusion, our data support the hypothesis that stem cells resident in adult cardiac tissues protects cardiac myocytes and fibroblasts from *T. cruzi* infection by secreting the antimicrobial peptide cathelicidin. This project may establish a new concept in stem cell biology as it is currently unknown whether CPCs express antimicrobial peptides, which are studied mostly in the context of neutrophils and other immunes cells and not in stem cells (as far as we are aware of). It will also reveal a new mechanism in innate immunity to *T. cruzi*, as the role of AMPs in *T. cruzi*-host interaction has not yet been studied. In addition to the mechanistic considerations summarized above, this project may reveal a novel agent to inhibit *T. cruzi* infection of cardiomyocytes in an *in vivo* situation, namely, for therapeutic application in chronic Chagas cardiomyopathy (CCC). CCC is the condition that kills Chagas disease patients, usually many years after infection and it is due to low levels of heart parasitism that infect and re-infect cardiomyocytes. Current anti-*T. cruzi* drugs (Nifurtimox and Benznidazole) cannot reduce low level parasitism in CCC, as indicated by many clinical trials. Cathelicidin highlights a new anti-*T. cruzi* compound that naturally blocks cardiomyocyte infection *in vitro*. As future plans focus on the continuation of this work, it will be exciting to determine if cathelicidin is active in a mouse model of CCC, by first studying disease progression in cathelicidin deficient mice, and later by systemic administration of the AMP.

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