RELEASE OF SOLUBLE INTERLEUKIN-7 α RECEPTOR (CD127) FROM $CD8^+$ T-CELLS AND HUMAN THYMOCYTES

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ABSTRACT

Background

Interleukin-7 (IL-7) is a cytokine crucial for T-cell development and homeostasis. IL-7 is thought to be a limited resource, and its interaction with the IL-7 receptor (IL-7R) has effects on increasing cell survival, proliferation and cytolytic function. Considering the roles of IL-7, it is no surprise that the expression of the IL-7 receptor alpha chain (CD127) is tightly regulated. Despite increased levels of soluble CD127 (sCD127) being detected in a number of disease states and being associated with disease activity, the biological function of sCD127 and its clinical relevance remains to be established. In this study, I explore the post-translational mechanisms leading to the release of the soluble form of CD127 receptor through IL-7 and αCD3/αCD28 stimulation. Here I specifically established two different mechanisms by which CD127 is processed; shedding of the receptor ectodomain and clipping.

Results

In $CD8^+$ T-cells, IL-7 plus TcR stimulation resulted in an increased release of sCD127. Here I found that matrix metalloproteases (MMPs), in particular MMP-9, have a role in the proteolytic clipping of CD127 resulting in the release of sCD127. In addition, I found that IL-7 plus TcR stimulation resulted in an increase in MMP activity and this activity was particularly dampened when MMP-2 and -9 inhibitors were used. I also found that neither MMP-3 nor cysteine and serine proteases seem to be directly involved in the generation of sCD127. Using a biotinylation assay I found that CD127 is being shed from the surface of $CD8^+$ T-cells as well as thymocytes through a MMP-independent mechanism.

Conclusion

These results demonstrate that MMPs (in particular MMP-9) have a role in the generation of sCD127. Further studies are required to determine the specific sheddase responsible for the ectodomain shedding of CD127, as well as the details behind the regulation of MMP-9 activity both in $CD8^+$ T-cells and thymocytes. A thorough understanding of these mechanisms will aid in the development of alternative and more specific strategies to control IL-7 mediated processes in both normal and disease states.

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ADAM	_	A disintegrin and metalloproteinase
α-	_	Anti
Ab	_	Antibody
APC	_	Antigen presenting cell
APS	_	Ammonium persulfate
Bcl-2	_	B-cell lymphoma 2 (anti-apoptotic)
BSA	_	Bovine serum albumin
C	_	Celsius
CD	_	Cluster of differentiation
CD127	_	IL-7 receptor alpha chain
CD132	_	Common gamma chain
CHEO	_	Children's Hospital of Eastern Ontario
CO_2	_	Carbon dioxide
CTL	_	Cytotoxic T-lymphocytes
Cys	_	Cysteine
DMSO	_	Dimethyl sulphoxide
DTT	_	Dithiotreitol
ECL	_	Enhanced chemiluminescence
ELISA	_	Enzyme linked immuno-sorbent assay
ECM	_	Extracellular Matrix
Em	_	Emission
Ex	_	Excitation
FBS	_	Fetal bovine serum
FRET	_	Fluorescence resonance energy transfer
g	_	Grams
x g	_	Force of gravity
HBV	_	Hepatitis B Virus
HCl	_	Hydrochloric acid
hr	_	Hour
HRP	_	Horseradish peroxidase
H_2SO4		Sulfuric acid
IFN-γ	_	Interferon-gamma
Ig	_	Immunoglobulin(s)
IL-	_	Interleukin
IL-7R	_	Interleukin 7 receptor

Jak	_	Janus associated kinase
Kb	_	Kilobase
KCl	_	Potassium chloride
kDa	_	Kilodalton
LPS	_	Lipopolysaccharide
mAb	_	Monoclonal antibody/antibodies
mCD127	_	Membrane form of IL-7 receptor alpha chain
MCL	_	Myeloid Cell Leukemia factor
mg	_	Milligram(s)
MHC II	_	Major histocompatibility complex II
min	_	Minute(s)
mL	_	Milliliter(s)
mM	_	Millimolar
MMP	_	Matrix metalloprotease
MS	_	Multiple sclerosis
μg	_	Microgram
μL	_	Microlitre
μM	_	Micromole
Na_2CO_3	_	Sodium carbonate
NaCl	_	Sodium chloride
NaF	_	Sodium fluoride
$NaHCO_3$	_	Sodium bicarbonate
$NaPO_4$	_	Sodium phosphate
NK	_	Natural killer
PBMCs	_	Peripheral blood mononuclear cells
PBS	_	Phosphate buffered saline
pg	_	Picogram(s)
PI3K	_	Phosphotidyl inositol-3-kinase
Pro	_	Proline
PVDF	_	Polyvinylidene difluoride
RPM	_	Revolutions per minute
RT	_	Room temperature
sCD127	_	Soluble form of IL-7 receptor alpha chain
SD	_	Standard deviation
SE	_	Standard error
SDS	_	Sodium dodecyl sulphate

SDS-PAGE	—	Sodium dodecyl sulphate poly acrylamide gel electrophoresis
Ser	—	Serine
SNPs	—	Single nucleotide polymorphisms
STAT	—	Signal transducers and activators of transcription
TBS	—	Tris buffered saline
TBST	—	Tris buffered saline and Tween-20
TcR	—	T cell receptor
TEMED	—	N,N,N’,N’-tetramethylethylenediamine
TGF-β	—	Transforming growth factor
Th	—	T-helper
Thr	—	Threonine
TIMPs	—	Tissue inhibitor of metalloproteinase
TLR	—	Toll-like receptor
TMB	—	3,3',5,5'-Tetramethylbenzidine
TNF-α	—	Tumor necrosis factor-alpha
U	—	Unit(s)
v	—	Volts
v/v	—	Volume with respect to volume
w/v	—	Weight with respect to volume

LIST OF FIGURES

CHAPTER 1: INTRODUCTION

1.1 T-cell maturation and activity

The immune system is a highly dynamic system that protects the host against invading pathogenic microorganisms [1]. Protection is achieved through a network of different cells and molecules that recognize and eliminate a variety of antigens [1]. When the immune system encounters a pathogen, different immune cells and molecules are recruited to the threat and mount the appropriate response to eliminate or neutralize the threat [1].

The immune system can be classified into two different components [2]. A nonspecific component, innate immunity, includes several resistance mechanisms against infection, but is not specific to a particular pathogen [2]. Some cells that are part of the innate immunity include neutrophils, basophils and eosinophils [1]. The specific component, adaptive immunity, is highly specific towards a pathogen and has the property of memory, allowing for a faster immune response when the host is re-infected by the same pathogen [2].

T-cells contribute to the immune defense of the host in two major ways: some T-cells function in directing and regulating immune responses; whereas other T-cells directly attack pathogen infected or cancerous cells [2, 3]. Helper T-cells, coordinate immune responses by communicating with other immune cells, to either produce antibodies or to activate other T-cells [3]. Cytotoxic T lymphocytes (CTL) directly attack other cells expressing certain foreign or abnormal antigens on their surfaces [2, 3].

T-cells are generated in the bone marrow and migrate into the thymus where they undergo maturation [3]. The thymus is the main site of T-cell development, however there is a small subset of cells that develop in secondary lymphoid organs [3]. Commitment to the T-cell lineage only happens once the cell has entered the thymus [4]. Once the bone marrow progenitor cells have entered the thymus, they are known as thymocytes [4, 5]. A critical step

in T-cell maturation is the rearrangement of the T-cell receptor (TCR) gene which involves the cutting and joining of variable, diversity and joining elements to create the rearranged TCR [4]. This rearrangement maximizes the repertoire of antigens recognized by the receptor, and thymocytes that fail to rearrange the TCR locus will die via apoptosis [4]. Successful rearrangement is confirmed by specialized positive and negative selection which results in a repertoire of cells that recognize self-major histocompatibility complex (MHC) proteins but not self-peptide [5]. Following selection, cells migrate into the thymic medulla where they mature into either single positive CD4 or single positive CD8 thymocytes [5]. Only 1-3% of thymocytes successfully complete development and leave the thymus to form the mature T-cell pool [5].

1.2 Cytotoxic T-Lymphocyte ($CD8^+$ T-cell)

$CD8^+$ T-cells, also known as cytotoxic T lymphocytes (CTL), play a key role in the cell-mediated response of the immune system [6]. The development, differentiation and activation of $CD8^+$ T-cells is fundamental for the successful clearance of tumor, virus and bacteria infected host cells [4]. $CD8^+$ cytotoxic activity is mediated through lytic and non-lytic pathways [4].

Two signals are required for the activation of $CD8^+$ T cells, which leads to the differentiation of naive lymphocytes into effector CTL [8]. T-cell activation requires recognition of specific antigenic peptides by the T-cell receptor (TCR), as well as additional co-stimulatory signals provided by accessory surface molecules on T cells [7, 8]. In the case where only the TCR-antigen signal is provided and the costimulatory signal is absent, lymphocytes will generally enter a state of anergy until further stimulatory signals are introduced [8]. This mechanism of regulation protects against the peripheral stimulation of

self-reactive T-cells, but can also result in immunologic tolerance to foreign and tumor antigens that are not presented properly by professional antigen presenting cells (APCs) [9, 10].

MHC class I displays peptide antigens that are generated from protein components of the intracellular pathogens (e.g. viruses) [6]. Antigens are generated through proteosomal degradation of foreign proteins in the APC's cytosol [6]. These peptide fragments are transported to the endoplasmic reticulum and complexed onto MHC class I molecules [6]. This MHC class I-peptide complex is then expressed on the cell surface where it is recognized by the TCR on specific $CD8^+$ T-cells [9]. To achieve proper activation of $CD8^+$ T-cells there needs to be co-stimulation through the CD28 surface molecule on $CD8^+$ T-cells [10].

Once $CD8^+$ T-cells become activated following interaction with a foreign antigen, they undergo rapid differentiation and proliferation to obtain an effector phenotype [10]. Currently there is controversy regarding how many times cells need to divide in order to differentiate into effector cells [9].

Once activated, $CD8^+$ T-cells kill infected cells primarily by secreting cytokines such as TNF-α and INF-γ, which have anti-tumor and anti-viral effects [9, 10]. Another mechanism of cell killing is via the production and release of cytotoxic granules, particularly perforin and granzymes [10, 11]. Perforins function is to make pores in the membrane of the infected cell and through this pore granzymes enter the target cell [11]. These granzymes are serine proteases that cleave proteins inside the infected cell and as a result the production of viral proteins is stopped and the target cell undergoes cell apoptosis [11]. To prevent the

damage of healthy bystander cells the cytotoxic granules are released only in the direction of the infected cell [11].

Another $CD8^+$ T-cell mechanism of killing consists of Fas/FasL interactions [11]. Activated $CD8^+$ T-cells express FasL on their cell surface, which binds to its receptor Fas expressed on the surface of the infected cell [11]. This binding causes a trimerization of the Fas molecules which activates a caspase cascade and induces apoptosis of the target cell [11].

Interleukin (IL) -7 stimulates proliferation of $CD8^+$ T-cells both in human and murine models in a dose-dependent manner [12]. Several studies have shown that IL-7 alone or in combination with IL-2 and IL-15 regulates the proliferation of memory $CD8^+$ T-cells [12, 13]. IL-7 is required to regulate the differentiation of effector $CD8^+$ T-cells into memory cells [14].

1.3 Interleukin-7

Interleukin 7 (IL-7) is a small glycoprotein (25kDa) that is essential for lymphocyte development and survival [15]. IL-7 was first discovered in 1988 as a factor that promoted the growth of murine B-cell precursors in a bone marrow culture system [15]. More recently, IL-7 was shown to be a T-cell growth factor and a regulator of T-helper cell (Th) 1 and 2 cytokine production, and able to stimulate differentiation of stem cells into lymphoid progenitor cells and promote survival and expansion of lymphoid precursors [16-18]. It has also been shown that IL-7 stimulates proliferation of cells of lymphoid lineage including T-cells and natural killer (NK) cells [19]. IL-7 is critical for survival and homeostatic proliferation of naïve T-cells, which has been shown by the reduced recovery of naïve

T-cells transferred into IL-$7^{-/-}$ mice and the impaired survival and homeostatic proliferation of T-cells from IL-7 receptor subunit (IL-7R)-deficient mice [20-23].

IL-7 is not produced by T-cells, but it is produced by a variety of other cell types including stromal cells in the thymus and bone marrow, vascular endothelial cells, intestinal epithelial cells, keratinocytes and follicular dendritic cells [24]. Furthermore, IL-7 increases cell numbers and the function of leukocytes by reducing apoptosis of activated T lymphocytes and promoting INF-γ [25].

IL-7 signaling has been shown to be critical for the development of T lymphocytes in the thymus, as well as controlling the size of the peripheral T lymphocyte population [26]. IL-7 signaling is involved in thymopoiesis, generation of mature T-cells, survival of naïve T-cells via up-regulation of Bcl-2 expression and the homeostatic expansion of naïve and memory T-cells via proliferation [27-28]. In addition, IL-7 augments the generation of anti-viral CTL and anti-tumor CTL activity [29].

1.4 Interleukin-7 receptor

IL-7 signals are transduced by the IL-7 receptor (IL-7R) which is composed of a unique alpha chain, CD127, and common gamma chain, CD132 [23]. When IL-7 binds to its cellular receptor it activates signal transducer and activator of transcription (STAT) 5 and phosphoinositide 3 kinase (PI3K) and leads to expression of Bcl-2 and Mcl-1 proteins, both of which promote naïve $CD8^+$ T-cell survival [23, 24]. Signaling by other γc cytokines, such as IL-2 and IL-4, increases cellular expression of their cognate receptors and results in $CD8^+$ T-cell proliferation and differentiation; whereas signaling by IL-7 reduces expression of its cognate receptor [30]. IL-7 induced CD127 downregulation has the beneficial effect of increasing the number of $CD8^+$ T-cells that can be supported by limiting amounts of *in vivo*

IL-7 [30-33]. Different T-cell subsets are stably maintained because IL-7-induced signals transiently downregulate IL-7R expression, ensuring that T-cell clones that have already received IL-7-induced survival signals do not express sufficient amounts of IL7R to compete for the remaining IL-7 with T-cell clones that have not yet received IL-7 induced survival signals [32, 33] In this way, the size of the peripheral T-cell pool is increased and the chance of survival of each naive T-cell clone is maximized [34].

The IL-7 receptor is a heterodimer composed of the cytokine specific high affinity, unique alpha chain (CD127) and the common gamma chain (CD132) [35]. CD132 is shared with other cytokine receptors such as IL-2, -4, -9, -15 and -21 [35]. Both chains are expressed independently on the cell surface and are associated through their cytoplasmic tails with JAK (JAK)- 1 which is associated with CD127 and JAK-3 which is associated with CD132 [35].

CD127 is a type I transmembrane protein which is composed of an extracellular domain, a transmembrane domain and an intracellular domain [36]. The extracellular domain is composed of two fibronectin type III subdomains [36]. The intracellular domain of CD127 consists of a Box 1 motif crucial for signal transduction through the Janus kinases, an acidic region, a serine-rich region and a distal region [36, 37].

IL-7 signaling through CD127 is essential for normal T-cell development and homeostasis [38]. Mice with CD127 deficiency show an early block in thymocyte development and reduced numbers of non-functional peripheral T-cells [38]. In humans, IL-7R-inactivating mutations result in severe combined immunodeficiency, whereas IL7R polymorphisms have been shown to confer susceptibility to multiple sclerosis (MS) [39-41].

There is also evidence that IL-7 and IL-7R may contribute to T-cell leukemia progression by stimulating proliferation of human leukemia cells as well as increasing cell viability [42].

IL-7 signaling is initiated after IL-7 binds to CD127, which then dimerizes with CD132 [35]. The interaction of the two receptor subunits brings the associated Jak-1 and Jak-3 into proximity and allows their subsequent trans-phosphorylation and activation [35]. This leads to phosphorylation of CD127 at a critical tyrosine residue (Y449) by Jak-1, allowing docking and subsequent phosphorylation and activation of signal transducer and activator of transcription (STAT) proteins and the p85 subunit of phosphoinositide 3 kinase (PI3K), both of which subsequently regulate the expression and activity of a number of downstream genes and gene products [35, 38]. Despite the central importance of IL-7 and its receptor in the development, maintenance and function of T-cell, the mechanisms regulating expression of the IL-7 receptor have only been partially characterized [19, 22, 32].

As cells differentiate from naïve to effector $CD8^+$ T cells, CD127 expression is down-regulated [23]. Due to the central role IL-7 has in this process, it is no surprise that aberrations in CD127 expression are associated with several disease processes including autoimmune diseases and different types of cancer [27]. For example, IL-7/IL-7R impairment contributes to failing $CD8^+$ T-cells responses and the development of chronic infectious diseases [43]. Decreased expression of CD127 on $CD8^+$ T cells in progressive HIV disease suggests a role for CD127 regulation in HIV immune-pathogenesis [44]. Furthermore, the frequency of naive, memory and effector memory $CD8^+$ T cells levels of CD127 on their surface in patients with multiple sclerosis (MS) are significantly higher than in healthy individuals [45]. A higher expression of the IL-7 receptor is also accompanied by augmentation of STAT5 phosphorylation in response to IL-7 signaling [45, 46]. Since

several single nucleotide polymorphisms (SNPs) have been linked to MS, multiple studies have been done to try and determine how mutations in CD127 contribute to MS disease state; however it remains unclear how these mutations contribute to MS [46].

The levels of CD127 expressed on T cells isolated from the joints of patients with rheumatoid arthritis (RA) where significantly higher compared to healthy controls as reviewed by Churchman *et al*. [47]. In one study where mice with RA were treated with antibodies against IL-7, researchers found that RA was eliminated; suggesting that aberrant IL-7 signaling has a role in development of RA [48].

In HIV-infected patients with uncontrolled plasma viremia, significantly fewer $CD8^+$ T-cells express cell surface CD127 compared to that in healthy individuals [49-52]. It is possible that the decline in $CD8^+$ T-cell activity in HIV infection is due to a decrease in the expression of the IL-7 receptor [51]. In HIV infection, decreased CD127 expression on T-cells is correlated with reduced $CD4^+$ T-cell counts, increased viral replication and immune activation [51].. Given the importance of IL-7 signaling in mediating $CD8^+$ T-cell function, CD127 down regulation may be one of the factors that significantly contributes to the $CD8^+$ T-cell dysfunction observed in HIV infection [43].

1.5 Soluble CD127

The molecular cloning of the human cDNA encoding IL-7 in 1989 by Goodwin *et al* was the starting point for a variety of further studies [36]. Several clones coding a soluble CD127 were isolated in addition to cDNA clones encoding the membrane-bound receptor [36]. Although IL-7 signaling is primarily regulated by membrane-bound CD127 (mCD127), recent data have also shown that a soluble form of CD127 (sCD127) may serve as a decoy

receptor to either suppress or enhance IL-7 signaling [30, 34]. There are several mechanisms through which soluble cytokine receptors can be produced with the two main mechanisms being: shedding of membrane-bound receptors and alternative splicing leading to a protein lacking the transmembrane domain [55]. Previous studies have demonstrated that sCD127 present in human plasma is primarily derived from alternative splicing and comprises an isoform lacking exon 6, as well as a unique 26-aa sequence that results from a frame shift and a premature stop codon [31].

Studies in our group have reported increased levels of sCD127 in HIV^+ plasma as compared to healthy controls and these levels correlate with increased levels of IL-7 [29, 30]. Based on these observations, one can hypothesize that the downregulation of surface CD127 may be attributed in part to the release of sCD127 by one or more cellular mechanisms and that this release may be driven by elevated levels of IL-7, chronic immune activation, or combination of these factors [29, 30]. The role of proteolytic cleavage as a mechanism of generation of sCD127 has yet to be studied and is the focus of this study.

1.6 Generation of soluble receptor

The majority of transmembrane proteins are also found in soluble forms that contain major parts of the extracellular domain [54]. This phenomenon has been observed for type I and II transmembrane proteins, both present in $CD8^+$ T cells [54]. Soluble receptors are conserved across species and several studies have demonstrated fundamental roles for soluble cytokines receptors in modulating cytokine activity [55]. The biological functions of soluble receptors can be quite different ranging from an antagonistic role like in the case of IL-1RII; to prolonging half-life as for sIL-6Rα; and potentiating signaling as described for sIL-15Rα [55]. Soluble cytokine receptors can be generated by several mechanisms, which

include proteolytic cleavage of receptor ectodomains, alternative splicing of mRNA transcripts, transcription of distinct genes that encode soluble cytokine-binding proteins, release of full-length receptors within the context of exosome-like vesicles, and cleavage of GPI-anchored receptors [55].

1.6.1 Proteolytic cleavage

Proteolytic processing at the cell surface and in the environment surrounding the cell by different proteases is emerging as an intriguing and novel level of control for the immune system [56]. Immune function depends on several events that activate and amplify the response targeted at the antigen, and proteolytic systems are crucial to all these events [56]. Two important families involved are the matrix metalloproteinases (MMPs) and the A Disintegrin Metalloproteinases (ADAMs) [56]. MMPs are involved mostly in aspects of immune-cell migration, membrane protein proteolytic cleavage and cytokine and chemokine function [57]. Two of the main types of proteolytic processing include clipping and shedding [58].

1.6.1.1 Clipping and shedding

Shedding is the proteolysis of the ectodomain of cell-surface proteins including chemokine, cytokine and growth factor, receptors as well as adhesion molecules [59]. Shedding can occur through different mechanisms via soluble or membrane-bound protease activity [59]. Ectodomain shedding is carried out by proteases of the ADAM family; MMPs and the aspartic protease, β-site APP- cleaving enzyme (BACE), resulting in the release of a peptide that often possess its own biological properties [59] . Shedding occurs at the surface of cell membranes and results in the release of the ectodomain (Figure 1A). The process of

shedding might initiate or dampen a signaling response depending on the stimulatory or inhibitory roles of the target being cleaved [59]. The different processing mechanisms are usually classified depending on the protease responsible for the specific process.

Clipping is defined as the proteolytic activation or inactivation of chemokines and other chemoattractants by the removal of short N- or C-terminal peptides [58]. Clipping can occur anywhere within the transmembrane domain of a receptor and cause the release of extracellular or intracellular soluble receptor fragments (Figure 1B) [58]. MMPs are particularly important players in the clipping mechanism; however aminopeptidases, carboxypeptidases and proteinases of other mechanistic classes have also been shown to have clipping activities in various cell types [58].

1.8 Proteases

Proteases are a class of enzymes that breakdown proteins by hydrolyzing the peptide bond [61]. All proteases share in common the general mechanism of a nucleophilic processing on the carbonyl-carbon of an amine bond [61]. Proteases are classified according to their catalytic site into four major classes: serine proteases, cysteine proteases, aspartic proteases and metalloproteases [62, 63].

1.8.1 Serine and cysteine proteases

Serine proteases represent one third of all known proteolytic enzymes [63]. Trypsin is the most genetically diverse among these proteases and is involved in a wide variety of functions [64]. Perhaps the most well-known are the trypsin-like enzymes of plasma, some of which play a central role in the reactions involved in the blood clotting [64]. Another example includes digestion in the intestine where trypsin in the duodenum catalyzes the hydrolysis of

peptide bonds to break down proteins into smaller peptides [64]. These enzymes share a common catalytic mechanism for selective cleavage of specific substrates and are frequently involved in protease cascades that involve a protease precursor being the substrate for an active protease [65]. This particular mechanism allows for a single signal to be amplified each time a downstream pro-enzyme (zymogen) is activated thereby increasing the proteolytic potential [65].

The membrane-anchored serine proteases are proving to be key components of the cell machinery for the activation of precursor molecules in the microenvironment surrounding the cell, with several crucial roles during development and maintenance of homeostasis [66]. Furthermore, there is growing evidence of their participation in the pathogenesis of inflammatory diseases [66]. Endogenous protein substrates targeted by membrane-anchored serine proteases include growth and differentiation factors, receptors, adhesion molecules and viral coat proteins [66].

Cysteine proteases are traditionally viewed as lysosomal mediators of terminal protein degradation [67]. However, recent findings suggest a more expanded role of these proteases in human biology [67]. Among these roles are apoptosis, MHC class II immune responses and extracellular matrix remodeling important to bone development [67]. Recent *in vitro* work has shown that Derp 1, a cysteine protease, selectively cleaves human CD25, the alpha subunit of IL-2 receptor to form soluble CD25 [68].

Figure 1. Schematic illustrating the site of proteolytic cleavage and products after cleavage for both clipping and shedding mechanisms.

The extracellular domain of the transmembrane protein is subject to proteolytic processing by one of a variety of different proteases. Protease-mediated cleavage of the transmembrane protein can result in the release of the ectodomain. **(A)** Illustrates the shedding mechanism resulting in release of receptor ectodomain; the middle panel indicates the proteolytic cleavage site at the surface of the cell membrane. **(B)** Illustrates the clipping mechanism resulting in release of receptor ectodomain; the middle panel indicates the proteolytic cleavage site somewhere within the transmembrane domain.

Fig. 1 (A)

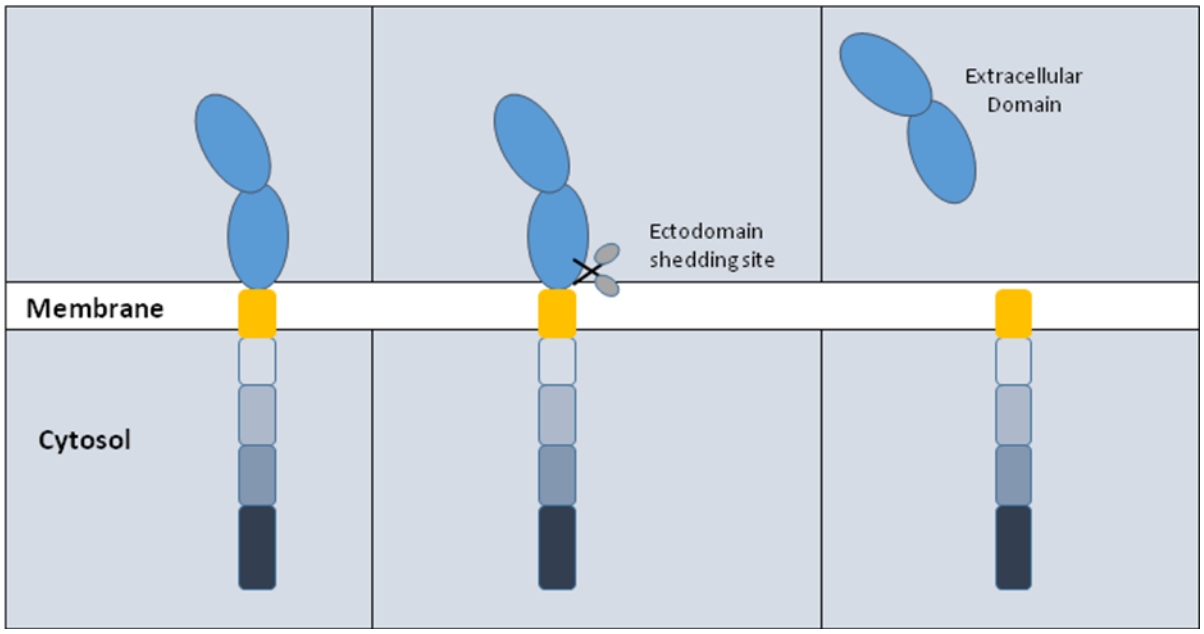
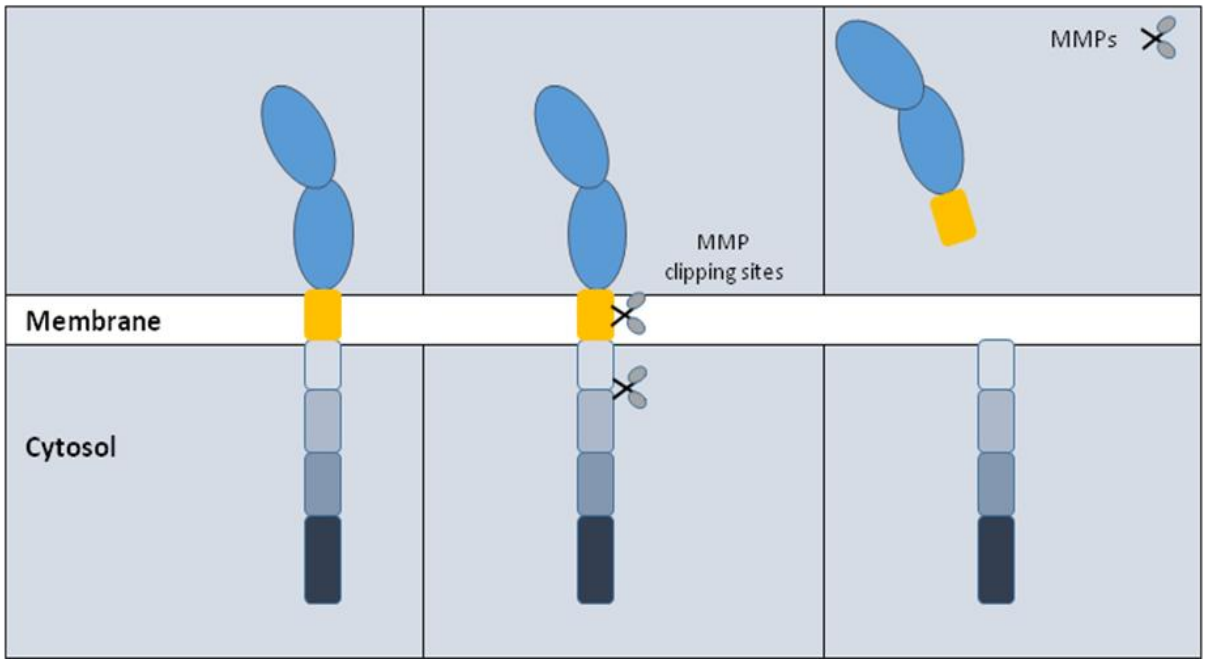

Extracellular
Domain
Ectodomain
shedding site
Membrane
Cytosol

Fig. 1 (B)


MMPs
MMP
clipping sites
Membrane
Cytosol

1.9 Matrix Metalloproteases (MMP)

Matrix metalloproteases constitute a family of over 20 different endopeptidases [69]. MMPs are members of the metzincin group of proteases which share the conserved zinc-binding motif in their catalytic active site; this characteristic is shared with the ADAMs [70]. MMPs are neutral endopeptidases produced as secreted or membrane bound pro-enzymes or zymogens, which become activated by removal of the NH_2-terminal propeptide [69]. The interaction of a conserved cysteine in the propeptide with the catalytic Zn^{2+} ion seals in the catalytic site and results in the latency of the pro-enzyme [70]. Removal of the propeptide, for example by proteolysis, causes conformational changes that allow the Zn^{2+} ion to become available for the binding of hydrolytic water molecule and of the substrate [69]. Due to these interactions the MMP activation mechanism has been named the "cysteine switch mechanism" which can be mediated by proteases and other MMPs [69].

Our knowledge of MMPs has evolved in the past few years, leaving behind the initial concept that proteases only act on the soluble phase, to recognizing all the different proteolytic reactions associated with the cell surface and the generation of soluble receptor, with which MMPs are involved [69]. MMP substrates include several different important cell surface molecules including adhesion molecules, apoptosis mediators, receptors, chemokines, cytokines, growth factors, proteases and structural molecules [72]. MMP-1,-2, -3, -7, -9 and -12 can release active TNF-α from the membrane-anchored precursor by a similar mechanism to TNF-α converting enzyme (TACE) which is also known as ADAM-17 [71]. MMPs can both activate pro-IL-1 β proteolytically and cleave the activated form of IL-1β to an inactive form, thereby providing both positive and negative regulation [72, 73]. MMPs also modulate chemokine activity [74]. MMP-9 has been shown to cleave CXCL8 (IL-8) to a fragment with 10 times the potency of the parent molecule [75]. Proteolysis of

cell surface proteins by MMPs may have extremely diverse biological implications, ranging from cell maturation and activation, to inactivation or degradation of substrates [69]. Modification of membrane-associated proteins by MMPs is crucial for communication between cells and the extracellular environment, maintaining the integrity of the tissue as well as determining the fate of cells [69].

MMPs enable normal cells to remodel the extracellular matrix (ECM), including basement membrane [69]. In healthy individual tissue, disruption is prevented by precise regulation of MMPs; however in cancer a number of MMPs are over expressed causing tissue disruption and making tumor cells capable of invasion and metastasis [74, 75]. Furthermore, it has been shown that MMP inhibition may affect T-cell migration and/or decrease cellular activation [75].

1.9.1 MMP-2 and MMP-9 (Gelatinases)

Gelatinases (MMP-2 and MMP-9) digest gelatin with the help of the three fibronectin type II repeats that binds to gelatin/collagen [75, 76]. They also digest a number of molecules in the extracellular matrix (ECM) including type IV, V and XI collagens, laminin, and aggrecan core protein [77]. MMP-2, but not MMP-9, digests collagens I, II and III in a similar manner to the collagenases [76]. The collagenolytic activity of MMP-2 is much weaker than MMP-1 in solution, but because proMMP-2 is recruited to the cell surface and activated by the membrane-type MMPs (MT-MMPs) it may express reasonable collagenolytic activity on or near the cell surface [76, 77].

The gelatinases, active on denatured collagens, incorporate three fibronectin type II repeats for the binding of gelatin; and MMP-9 is the only MMP to possess a Ser/Thr/Pro-rich

O-glycosylated domain which forms an attachment site for multiple O-linked sugars [78]. MMP-2 is thought to be constitutively expressed whereas the expression of MMP-9 needs to be induced [79]. The induction of MMP-9 is thought to be regulated by various factors, such as cytokines and growth factors [79]. Among these stimuli, it is of particular interest that both TNF-α and IL-1 stimulate MMP-9 production in macrophages, endothelial cells, connective tissue cells and T-cells [80].

MMP-2 and -9 have been shown to be involved in T-cell infiltration into tissues suggesting a role for them in T-cell mediated injury [81]. Furthermore, studies have shown that $CD4^+$ and $CD8^+$ T cells can produce MMP-2 and MMP-9 upon stimulation [81, 82]. Different studies have demonstrated a direct role for MMPs, in particular 2 and 9, in T-cell activation [82]. In one study, MMP-9 deficient mice had significant impairment in the activation of $CD4^+$ and $CD8^+$ T-cells [82].

1.9.2 Regulation of MMPs activity

MMPs are important regulators of tissue homeostasis and immunity, they are particularly crucial for communication within tissues and cells [83]. Due to the fact that uncontrolled MMP activity can easily become destructive and lead to breakdown of homeostasis, their activity is tightly regulated [83].

MMP activity is regulated at four different levels: 1) gene expression with transcriptional and post-transcriptional regulation; 2) extracellular localization and compartmentalization; 3) pro-enzyme activation by removal of the pro-domain; and 4) inhibition by specific inhibitors, i.e. tissue inhibitors of matrix metalloproteinases (TIMPs) and by non-specific proteinase inhibitors, i.e. a2-macroglobulin [84]. Once active, MMPs

can modulate the global proteolytic potential in the extracellular milieu through zymogen (MMP pro-form) activation and inhibit degradation or inactivation of other proteases [70].

1.9.3 MMPs in disease

While appropriate MMP secretion facilitates an effective immune response, host tissue damage may be caused by excess MMP activity resulting from either increased MMP secretion or decreased TIMP secretion [84]. Proteolysis in the extracellular matrix is a key component of the inflammatory process and MMPs are crucial for this process [84]. Thus, they are considered important constituents in the host response to infections, trauma, autoimmune and toxic conditions [84].

MMPs have also been found to have an important role in pathogenesis of several cardiovascular diseases including cardiomyopathy, atherosclerosis, congestive heart failure, restenosis, myocardial infarction (MI), and aortic aneurysm [81]. Another pathological area where MMPs play a key role is in tumor progression [81]. Tumor progression and metastasis require the breakdown of the ECM by release of enzyme such as MMPs to enable invasion of abnormal cells [81]. In certain cases, this invasion of cells is radically affected by the levels of MMPs. TIMPs are endogenously expressed inhibitors of MMPs, and can decrease metastasis of tumor cells [81].

HIV dementia is a devastating complication of chronic HIV infection, characterized by neuronal death accompanied by the invasion of activated macrophages and microglia [85]. HIV infection of monocytes increases MMP-9 secretion in vitro, the activity of which results in increased endothelial cell monolayer permeability, which may explain the increased blood-brain barrier permeability that occurs in HIV infection [85]. Soluble HIV-

Tat protein alone can up-regulate monocyte MMP-9 secretion and HIV-1 envelope glycoprotein 120 increases MMP-9 secretion by T cells and glioma cells [86, 87].

The hepatitis B virus (HBV) X protein induces MMP-14 expression and this correlates with increased invasiveness of HBV *in vitro* and *in vivo* which can result in liver cancer [88]. MMP-14 may increase invasiveness of HBV by a directly affecting the ECM, but also by activating MMP-2 which could also be affecting the ECM, this is a clear example the complexity of proteolysis regulation [88]. In acute HBV infection, excessive inflammation leads to necrosis, with the recruitment of antigen-nonspecific lymphocytes to the liver [89]. This recruitment is associated with MMP-2 and -9 expression [89].

Endotoxic shock results from a severe, generalized inflammatory response induced by bloodstream infection with gram negative bacteria [90]. Organ failure is associated with increased vascular permeability of which MMPs may contribute to this breakdown of the endothelial barrier [91]. Bacterial lipopolysaccharide (LPS) is known to up-regulate MMP-1, -7, and -9 secretion by monocytes and macrophages and MMP-9 release by neutrophils [90, 92].

Peptic ulcer disease and gastric cancer associated with chronic *H. pylori* infection, both involve abnormal breakdown to the ECM [93]. *H. pylori* infection results in an increase in MMP-7 expression *in vivo* and *in vitro* studies have shown that cell infection increases MMP-7 secretion [93].

1.9.4 MMP inhibitors

MMP inhibitors can be classified as either endogenous or exogenous, depending on their source [94]. The most commonly found endogenous MMP inhibitors are TIMPs and they can be classified into four major proteins: TIMP-1, -2, -3 and -4 [94]. These inhibitors act by binding to the catalytic domain of MMPs, thereby inactivating them [94, 95]. In the case of gelatinases, the proenzyme creates a complex with TIMPS which prevents them to transforming to their active form [95]. TIMPs are composed of a large N-terminal domain responsible for MMP inhibition and a smaller C-terminal domain [95]. The transcription of TIMPs is regulated by cytokines and growth factors in a similar way as MMP expression is regulated [94].

1.9.4.1 Synthetic inhibitors structure

Exogenous MMP inhibitors are synthetic. Most MMPs inhibitors consist of two parts, a zinc binding group and a backbone [96]. Hydroxamic acids are among the most common synthetic MMPs inhibitors [96]. They can form hydrogen bonds with several residues within the MMP active site [97]. Despite the fact that these inhibitors work efficiently in vitro, they have not been successful during clinical trials [98].

Rationale:

Despite the crucial role of IL-7 in the development, maintenance and overall function of T cells, the mechanisms regulating the expression of IL-7 receptor have only been partially characterized. IL-7 downregulates the expression of the CD127 receptor subunit on the surface of T cells [31]. However the mechanisms behind this downregulation are still unclear. Some studies have reported transcriptional suppression of the CD127 gene in response to IL-7, while others have found IL-7 does not affect CD127 mRNA levels in human T cells [49]. In addition to this, our group has shown that CD127 downregulation is in part due to the shedding of the receptor from the cell surface in the form of sCD127. Naive $CD8^+$ T cells are more sensitive to IL-7 mediated down-regulation of CD127, suggesting that these effects may have particular significance early in T-cell life cycle; because of this we decided to investigate the regulation of sCD127 on human thymocytes as well [31,32].

Due to the conflicting data regarding this phenomenon we sought to determine the specific mechanisms behind the generation of sCD127 and the relation with the downregulation of CD127. In this study, I explore two different mechanisms by which sCD127 can be generated and discuss the potential implications of this release in T-cell homeostasis.

Hypothesis:

Release of soluble CD127 by $CD8^+$ T-cells and human thymocytes is induced by cytokine-TcR stimulation.

Objectives:

1. To determine the mechanisms mediating the release of sCD127 in $CD8^+$ T-cells.

    a) Determine the specific proteolytic mechanisms leading to the release of sCD127.

2. To determine the mechanisms mediating the release of sCD127 in thymocytes.

    a) Determine the specific proteolytic mechanisms leading to the release of sCD127.

CHAPTER 2: MATERIALS AND METHODS

2.1 Reagents

2.1.1 Stimulation reagents

Recombinant human IL-7 (I5896; Sigma-Aldrich Inc, Saint Loius, MO, USA) was reconstituted at 10 μg/ml in distilled water. Purified functional mouse anti-human CD3 (α-CD3) (14-0038; eBioscience, San Diego, CA, USA) and mouse monoclonal anti-human CD28 (α-CD28) (16-0289; eBioscience) antibodies were purchased pre-dissolved in aqueous buffer at a concentration of 1 mg/ml. IL-7 was aliquoted and stored at -20° Celsius (C) and the α-CD3 and α-CD28 were stored at 4°C.

2.1.2 ELISA reagents

Recombinant human CD127-Fc chimeric protein (306-IR; R&D Systems, Minneapolis, MN, USA), used to make the standard curve, was reconstituted at 100 μg/ml in phosphate buffered saline (PBS; Gibco, NY, USA). Mouse monoclonal anti-human CD127 capture antibody (MAB4774; R&D Systems) was reconstituted at 500 μg/ml in PBS. Biotinylated mouse anti-human CD127 detection antibody (BAM47741; R&D Systems) was reconstituted at 50 μg/ml in Tris-buffered saline (TBS; pH 7.3) containing 20 mM Trizma base (T1503; Sigma-Aldrich Inc.), 150 mM sodium chloride (NaCl; Fisher Scientific, NJ, USA) and 0.1% bovine serum albumin (BSA; A2153, Sigma-Aldrich Inc.). Streptavidin horseradish peroxidase (HRP) conjugate (SA202; Millipore, CA, USA) was obtained pre-dissolved at a concentration of 1 mg/ml in 0.05 M $NaHCO_3$ (pH = 8.3) containing 0.05% thimerosal. All reagents were aliquoted and stored at -80° C except for streptavidin-HRP which was stored at 4° C.

2.2 Cell Culture

2.2.1 Human $CD8^+$ T-cells

All research conducted using blood from human subjects was approved by the Ottawa Hospital Research Institute Ethics Board. Peripheral blood was collected in 100 units (U)/ml heparin (LEO Pharma Inc., ON, Canada) from HIV seronegative subjects. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque™ PLUS (GE Healthcare Bio-Sciences AB, Sweden) density gradient separation by centrifuging at 1600 rpm for 30 minutes with no brake using a Heraeus Instruments centrifuge (Megafuge 1.0, Heraeus Instruments, Germany) and subsequently washed twice with PBS (Gibco). Washed PBMCs were re-centrifuged and resuspended at a concentration of 1×10^8 cells/ml in PBS containing 5 g/L BSA (Sigma-Aldrich Inc.) and 2 mM ethylenediaminetetraacetic acid disodium salt (EDTA; Fisher Scientific; pH 8). $CD8^+$ cells were isolated from PBMCs using $CD8^+$ Positive Selection Kit (Stemcell Technologies Inc., BC, Canada) in accordance with the protocol provided by the manufacturer. Purified $CD8^+$ T-cells were counted as previously described and resuspended at a concentration of 2×10^6 cells/ml in 1X RPMI 1640 media (Gibco) supplemented with 20% heat inactivated fetal bovine serum (FBS; 26140-087; Gibco), 100 µg/ml penicillin-streptomycin (10378-016; Gibco) and 0.25 mM L-glutamine (25030-081; Gibco).

2.2.1.1 $CD8^+$ T-cell Stimulation

Following positive isolation as described above cells were rested overnight at 37°C in a HEPA Class 100 Stericycle CO_2 Incubator (Thermo Electron Corporation, OH, USA) to recover baseline CD127 surface expression, which is transiently downregulated upon

isolation. The following day, $CD8^+$ T-cells were washed, recounted and resuspended at 2 x 10^6 cells/ml in fresh RPMI1640 media supplemented with 20% FCS. $CD8^+$ T-cells were then stimulated at a concentration of 1 x 10^6 cells/ml with the combination of IL-7 (10 ng/ml) plus α-CD3/α-CD28 (1μg/ml). Unstimulated $CD8^+$ T-cells were used as a media control. Stimulated and unstimulated $CD8^+$ T-cells were incubated for 72 hours at 37° C. The 72 hour time point was established as the peak of sCD127 release by previous optimization protocols. Following incubation, $CD8^+$ T-cell culture supernatants were collected in siliconized microtubes (Sigma-Aldrich, Inc.) and stored at -80°C until ready to be analyzed by a sCD127-specific ELISA.

2.2.2 OP9-DL1 cell line

OP9-DL1 cells were provided by Dr. Zúñiga-Pflücker (University of Toronto). Cells were maintained in minimal essential medium-alpha (MEM-α; 12561-072; Gibco) supplemented with 100 μg/ml penicillin-streptomycin (Gibco) with 20 % FBS (Gibco). Cells were maintained in a single monolayer and then trypsinized and reseeded at a density of 0.2 x 10^6 cells/ml every 2-3 days. Cells were cultured at 37°C in a HEPA Class 100 Stericycle CO_2 Incubator (Thermo Electron Corporation)

2.2.3 Thymocytes

Thymic tissue was obtained during elective cardiac surgery at the Children's Hospital of Eastern Ontario (CHEO) with informed consent obtained prior to surgery. This study was approved by the CHEO Research Ethics Board. Thymic tissue was washed twice with warmed PBS. The thymus was submerged in PBS and connective tissue surrounding the thymic organ was removed. Thymic tissue was cut into 1-3 mm^3 pieces with a scalpel and

homogenized with the plunger of a 60 cc syringe. The homogenized thymus tissue was layered on a Ficoll-Paque^{TM} PLUS (GE Healthcare Sciences AB, Sweden) density gradient and thymocytes were isolated following the protocol for isolating PBMCs. The isolated cells were resuspended in McCoys 5A selective medium (16600-108; Gibco) supplemented with 2mM glutamine (Gibco), 100 µg/ml penicillin-streptomycin (Gibco) and 10% FBS (Gibco). Cells were stored at 4°C prior to use; optimal use of thymocytes is within one week. Thymocytes that were to be used were co-cultured with a monolayer of OP9 DL1 cells at 37°C in a HEPA Class 100 Stericycle CO_2 Incubator (Thermo Electron Corporation).

2.2.4 Treatment of cells with pharmacological inhibitors

To determine the effect that Jak-1, STAT5 and PI3-K had on IL-7 plus TcR induced release of sCD127, inhibitors specific to these signaling molecules were used. Jak-1 inhibitor (420099; Calbiochem, La Jolla, CA), STAT5 inhibitor (573108; Calbiochem) and PI3K inhibitor (LY294002; Calbiochem) were resuspended each in dimethyl sulphoxide (DMSO) to 10mM, aliquoted and stored at -20°C. To determine which protease is involved in the release of sCD127, multiple inhibitors were used. Broad MMP inhibitor, GM6001 (364205; Calbiochem) was dissolved in DMSO to 10mM. Broad MMP plus ADAM17 inhibitor, TAPI-0 (579050; Calbiochem) was dissolved in DMSO to 10mM. MMP-2/9 inhibitor (444241; Calbiochem) was dissolved in DMSO to 10mM. MMP-9 inhibitor (444278; Calbiochem) was dissolved in DMSO to 10mM. MMP-2 inhibitor (444294; Calbiochem) was dissolved in DMSO to 10mM. MMP-3 inhibitor (444218; Calbiochem) was dissolved in DMSO to 10mM. Cysteine protease inhibitor, leupeptin (108976; Calbiochem) was dissolved in distilled water at 1mg/mL. Serine protease inhibitor, aprotinin (A6279; Sigma) was pre-dissolved in 0.9% NaCl/0.9% Benzyl alcohol solution to 2.2mg/mL. All inhibitors

except aprotinin were aliquoted and stored at -20°C; while aprotinin was aliquoted and stored at 4°C.

Cells were left either untreated or treated with various concentrations of JAK inhibitor, STAT5 inhibitor and PI3-K inhibitor for 2 hours followed by stimulation with IL-7 plus α-CD3/α-CD28 for 72 hours. Supernatants were then analyzed by Western blot or ELISA. The viability of cells treated with each inhibitor was assessed by Trypan blue exclusion. Prior to use, the inhibitors were diluted in either PBS or DMSO and appropriate vehicle controls were included in all experiments.

2.3 Soluble CD127 protein measurement by ELISA

Concentration of sCD127 in $CD8^+$ T-cell culture supernatants was determined by sandwich ELISA as developed by Hoe et al [27]. High-binding, flat bottom 96 well plates (R&D Systems) were coated with 5 μg/ml of capture antibody dissolved in 0.2 M sodium carbonate in 500 ml distilled water, pH 9.4 (Thermo Scientific, IL, USA) and incubated at 4° C overnight. The next day, wells were washed with PBS and then blocked with PBS containing 3% (w/v) BSA and 0.1% (v/v) Tween-20 (Fisher Scientific) for 1 hour at room temperature. Next, $CD8^+$ T-cell culture supernatants were loaded as well as a standard curve consisting of CD127-Fc chimera at the following concentrations 1000, 500, 250, 125, 62.5, 0 pg/ml dissolved in PBS containing 1% (w/v) BSA. Culture supernatant from WI-26VA4 cells, a fibroblast cell line known to secrete CD127, was used as a positive control for the assay. All samples were loaded as triplicates and incubated at room temperature for 2 hours, after which wells were washed with PBS containing 0.05% (v/v) Tween-20 and loaded with 0.2 μg/ml of detection antibody dissolved in PBS containing 0.1% (w/v) BSA and incubated for another 2 hours at room temperature. After washing, wells were loaded with 1 μg/ml of

streptavidin-HRP conjugate dissolved in PBS containing 0.1% (w/v) BSA and incubated for 30 minutes at room temperature. Following a final wash, wells were loaded with tetramethylbenzidine (TMB) substrate (SurModics, MN, USA) to determine protein concentration. The reaction was stopped with 0.5 M sulphuric acid (H_2SO_4) (Sigma-Aldrich Inc.). Plates were read using a SpectraMAX 190 microplate spectrophotometer (Molecular Devices Corp., CA, USA) and SoftMAX Pro 2.4.1 software (Molecular Devices Corp., CA, USA) at 450 nm and values were corrected by subtracting measurements at 540 nm. Protein concentration was calculated by interpolating values from the standard curve using Microsoft Office Excel 2007 (Microsoft Corporation, ON, Canada) and outlier triplicates were excluded from the analysis based on a deviance of ± 0.04 from the median optical density value.

2.4 Biotinylation assay

$CD8^+$ T-cells samples of $2x10^6$ were labeled with biotin following the protocol provided with the Pierce Cell Surface Protein Isolation Kit (89881; Thermo Scientific, USA), stimulated as previously described (section 2.2.1.1) and incubated at 37° C for 72 hours. Culture supernatants were collected and biotinylated proteins were recovered using a NeutrAvidin® Agarose gel column in accordance with the instructions provided in the kit, whereas cell pellets were conserved for subsequent cell lysis analysis. Eluted biotinylated proteins were resuspended in denaturing SDS-PAGE loading buffer, 2X Laemmelli Buffer (BioRad, ON, Canada), and dithiothreitol (DTT; Thermo Scientific) and concentrated using Amicon® Ultra centrifugal filters (MWCO = 30000 kDa; Millipore) and an Eppendorf Centrifuge 5415 C (Brinkmann Instruments Inc., NY, USA). Concentrated ultrafiltrates were

collected in siliconized microtubes and stored at -20° C until ready to be assayed by Western Blot.

2.5 Western Immunoblot Analysis

2.5.1 Preparation of cell lysates

1×10^6 cells were seeded in each well of a 24 well plate. Each well was treated with a specific concentration of an inhibitor for 2 hr. Following this 10 ng/ml of IL-7 plus 1 µg/mL of α-CD3/α-CD28 was added to each well. Plates were incubated at 37°C and 5% CO_2 for 72 hours. Cell pellets were collected by spinning down the cell suspensions at 1600 rpm for 5 min and removing the supernatant. Cell pellets were lysed by adding 60 µl of complete lysis buffer and left to incubate for 1 hour on ice. Complete lysis buffer consists of protease inhibitor, 20mM Tris HCl (Sigma-Aldrich, Inc.), 150 mM NaCl (BDH, West Chester, PA) and 1 mM sodium orthovanadate (Sigma-Aldrich, Inc.). Lysates were spun down for 20 min at 20,000 x g at 4°C and then analyzed using a Bradford assay to determine protein concentration.

2.5.2 Bradford Assay and sample preparation

A standard was prepared by serial dilution of stock BSA (2 mg/ml) (Calbiochem). 2µl of each lysate was added to 198 µl of PBS. 200 µl of standards and samples were added to 96 well plate together with 50 µl of Protein Assay Dye Reagent Concentrate (Biorad, Mississauga, Ontario). Plates were read at 595nm using SpectraMAX 190 microplate spectrophotometer and SoftMAX Pro 2.4.1 software (Molecular Devices Corp.). After determining the protein concentration of each sample, 10µl of loading buffer (Biorad) was added to 30µg of protein lysate. Samples were boiled for 5min and then loaded into SDS-

PAGE.

2.5.3 Polyacrylamide gel electrophoresis (PAGE)

Proteins were resolved on 10% SDS-PAGE resolving gel. The resolving gel was composed of 5.9 ml distilled water, 5 ml 30% Bis-Acrylamide solution (Biorad), 3.8 ml resolving buffer, 150 µl of 10% SDS (Calbiochem), 150 µl of 10% Ammonium Persulfate (APS; Biorad) and 6 µl TEMED (Biorad). The resolving buffer was prepared by adding 91 g of Tris Base (Sigma-Aldrich, Inc.) and 2 g of SDS (Sigma-Aldrich, Inc.) to 300 ml distilled water, pH adjusted to 8.81, and bringing the final volume to 500 ml with distilled water. The 12% stacking gel was composed of: 3.4 ml distilled water, 830 µl 30% Bis-Acrylamide solution (Biorad), 630 µl stacking buffer, 50 µl of 10% SDS (Sigma-Aldrich, Inc.), 50 µl of 10% APS (Biorad) and 5µl TEMED (Biorad). The stacking buffer was prepared by adding 60.6 g Tris Base (Sigma-Aldrich, Inc.) in 300 ml distilled water, pH adjusted to 6.8, and bringing the final volume to 500 ml with distilled water. Pre-stained broad range protein markers (Bio-Rad) were used to estimate molecular mass of proteins. Electrophoresis was carried out in 1X running buffer at 120 volt for approximately 1.5 hours. Stock 5X running buffer was prepared by mixing 151 g Tris Base (Sigma-Aldrich, Inc.) and 72g Glycine (Biorad) in 1L of distilled water.

2.5.4 Electrophoretic transfer

Resolved proteins were transferred from the gel to a Polyvinylidene Fluoride (PVDF) membrane (Biorad) at 100 volts for 1 hourr, using 1X Transfer buffer. Stock 10X transfer buffer was prepared by mixing 60g Tris Base (Sigma-Aldrich, Inc.) and 72g Glycine (Biorad) in 1L of distilled water.

2.5.5 Immunoblotting

The membrane containing transferred proteins was incubated with a primary antibody (Ab), mouse monoclonal IgG anti-human CD127 antibody (R34.34 clone; Fisher Scientific), and overnight at 4°C. The primary Ab solution was prepared by dissolving 10µl of primary Ab in 10ml of PBS with 2.5% (w/v) BSA solution. The membrane was washed for 10 min, three times in 1X TBST buffer. Stock 10X TBST buffer was prepared by mixing 24.2g Tris HCl (Sigma-Aldrich, Inc.) and 80g NaCl (Sigma-Aldrich, Inc.) with 10ml Tween 20. Then 2.5µl of secondary Ab, a goat anti-mouse IgG HRP conjugate (HAF007; R&D Systems) was diluted in 10ml of blocking buffer consisting of 1X TBST and 5% skim milk (No Name, ON, Canada) and placed on membrane for 1 hour at room temperature on a belly dancer (Stovall Life Science, Greensbora NC, USA). The membrane was washed for 10 min, three times in 1X TBST buffer. The membrane was then placed in an enhanced chemiluminescence (ECL) solution (RPN2232; GE Healthcare, Buckinghamshire, UK) and visualized using a FluorChem® HD2 chemiluminescent imager (Alpha Innotech Corporation, CA, USA) and Alpha View 3.4 software (Alpha Innotech Corporation). Western Blot data was analyzed using AlphaEase® FC 6.0 software (Alpha Innotech Corporation). Membranes were stripped at 50°C for 20min in stripping buffer. The stripping buffer was prepared by adding 47.2g Tris HCl (Sigma-Aldrich, Inc.) and 10g SDS (Sigma-Aldrich, Inc.) in 500ml distilled water and adding 3.65ml of β-mercaptoethanol (Sigma-Aldrich, Inc.). To ensure equal loading of proteins the membranes were stripped and re-probed with mouse monoclonal anti-human β-actin antibodies (13-9923; e-bioscience).

2.6 Measurement of MMP protein levels by ELISA

MMP-9, MMP-2 and MMP-3 in supernatants were quantified by ELISA specific to each MMP (MMP-9 ELISA Kit, KHC3061; MMP-2 ELISA Kit, KHC3081; MMP-3 ELISA Kit, KAC1541; Invitrogen, Camarillo CA). In all cases, ELISA was carried out according to vendor's recommendation and the linearity of the assay confirmed for MMP-9 concentrations within 0 to 1500 pg/mL, for MMP-2 concentration between 0 to 20ng/ml and for MMP-3 concentration between 0.78 to 25ng/ml. In the case of MMP-9 ELISA, $CD8^+$ T-cells and thymocytes supernatants were diluted (1:1000) to attain an appropriate concentration within the linear range and loaded in duplicate in a 96-well format. MMP-2,-3,-9 protein was quantified via colorimetric detection using a microplate reader.

2.7 Assessing MMP activity by fluorometric activity assay

Culture supernatants and cell lysates from stimulation experiments previously described (section 2.2.1.1) were analyzed using Abcam MMP Activity Assay Kit (AB112146; Abcam, San Francisco, CA, USA). Samples were incubated with 1mM APMA at 37° as follows: MMP-2 for 1hr, MMP-3 for 24hr and MMP-9 for 2 hr. APMA incubation was used to activate all the MMPs present in the sample This kit uses a fluorescence resonance energy transfer (FRET) peptide as a generic MMP activity indicator. It is designed to determine the general activity of an MMP enzyme and to screen MMP inhibitors. In the intact FRET peptide, the fluorescence of one part is quenched by another. After cleavage into two separate fragments by MMPs, the fluorescence is recovered. The signal was read by a fluorescence microplate reader (LumiStar Optima Plate Reader, BMG Labtech, Germany) at Ex/Em = 490/525 nm.

2.8 Statistical analysis

All statistical analyses were conducted using GraphPad Prism 5.0 software (GraphPad, CA, USA). Analyses of variance over time were conducted using one-way ANOVA and Dunnett's multiple comparison tests, whereas comparisons of sCD127 concentration at a single time point were conducted by student t -test with $p \leq 0.05$ considered as a significant difference.

CHAPTER 3: RESULTS

3.1 Mechanism of sCD127 release from $CD8^+$ T-cells

Initial reports from our lab were aimed at describing how IL-7 downregulates the expression of CD127 on $CD8^+$ T-cells and the potential implications of this regulation. Further studies were focused on determining the mechanisms responsible for this downregulation and its relationship to the generation of sCD127 [29, 30]. Subsequent work suggested that shedding is one potential mechanism by which sCD127 is released [31]. Proteolytic cleavage has been suggested as a possible mechanism for this sCD127 release [31]. This initial work indicated that stimulation of $CD8^+$ T-cells with IL-7 plus α-CD3/α-CD28 antibodies induced the optimal release of sCD127.

3.1.1 Proteolytic cleavage on the release of sCD127

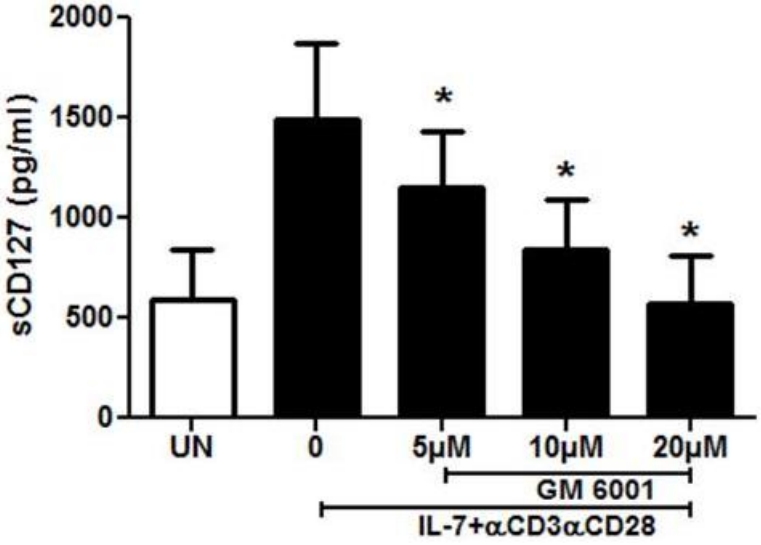
3.1.1.1 The effect of MMPs

To determine the role of proteolytic cleavage in the release of sCD127, stimulated $CD8^+$ T-cell were pre-incubated with a variety of proteases inhibitors. $CD8^+$ T-cells were first cultured in the presence of a broad MMP inhibitor, GM6001 (5, 10, 20 μM) and in media alone. These cells were then stimulated with a combination of IL-7 (10 ng/ml) and α-CD3/α-CD28 antibodies (1μg/ml). Supernatants were collected after 72 hours and the concentration of sCD127 was quantified by ELISA. Figure 2 shows that with increasing concentration of broad MMP inhibitor there was a significant reduction in the amount of sCD127 present in the supernatants of $CD8^+$ T-cells stimulated with IL-7 and α-CD3/α-CD28 antibodies.

Figure 2. Broad inhibition of MMP's reduces the amount of sCD127 released from stimulated $CD8^+$ T-cells.

$CD8^+$ T-cells were cultured in the presence of 5, 10, 20 μM of GM6001 or media alone for 2 hours, and then stimulated with a combination of IL-7 (10ng/ml) and α-CD3/α-CD28 antibodies (1 μg/ml each). Supernatants were collected after 72 hours and the concentration of sCD127 was quantified by ELISA. Bars represent mean ±SEM (n = 6). *p=0.0287 by One-way ANOVA and $p< 0.05$ by pairwise Dunnett test versus control (no inhibitor).

Fig.2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sCD127 (pg/ml)
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5μM
10μM
20μM
GM 6001
IL-7+αCD3αCD28

TAPI-0 is another potent broad inhibitor of MMPs which, in addition is also a potent inhibitor of ADAM17. $CD8^+$ T-cells were pre-treated with various concentrations of TAPI-0 (5, 10, 20 µM) and then stimulated with a combination of IL-7 (10ng/ml) and α-CD3/α-CD28 antibodies (1µg/ml); after 72 hours post stimulation the release of sCD127 was significantly reduced in stimulated cells treated with TAPI-0 compared to stimulated cells that were untreated (Figure 3).

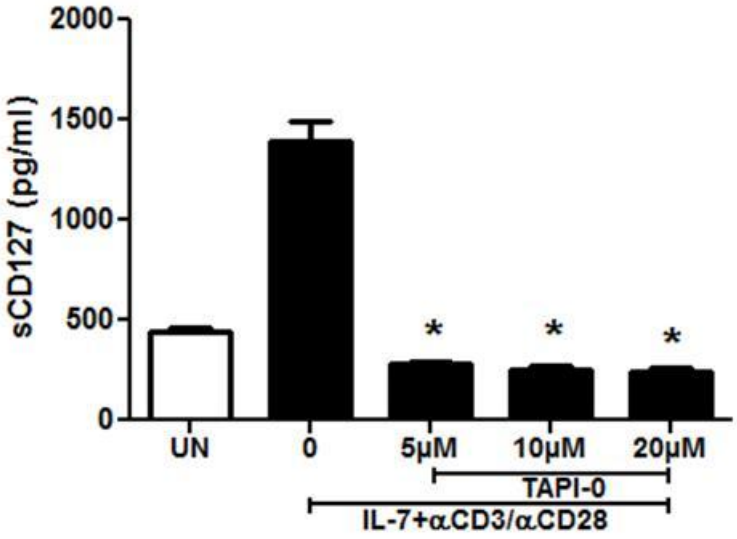
After determining that MMP's have a significant role in the release of sCD127, the goal was to determine which specific MMP was responsible for the release of sCD127. Using specific inhibitors for MMP-2/9 (10, 20, 50 µM) and MMP-3 (10, 20, 50 µM), there was a significant reduction in the release of sCD127 in stimulated $CD8^+$ T-cells treated with MMP-2/9 inhibitor (Figure 4A); whereas there was no significant reduction in sCD127 release after treatment with MMP-3 inhibitor (Figure 4B). Therefore it was determined that MMP-2/9, but not MMP-3 were responsible for the clipping of CD127 from $CD8^+$ T-cells.

Finally, specific inhibitors for MMP-2 (10, 20, 50 µM) and MMP-9 (10, 20, 50 µM) were used on stimulated $CD8^+$ T-cells. As seen in Figure 5A, inhibition of MMP-9 significantly reduced the release of sCD127; while inhibition of MMP-2 had no significant effect on the release of sCD127 from stimulated $CD8^+$ T-cells (Figure 5B).

Figure 3. TAPI-0, an inhibitor of both MMPs and ADAM17 (a TNF-α processing enzyme) causes significant reduction in the release of sCD127 from $CD8^+$T-cells.

$CD8^+$ T-cells were cultured in the presence of 5, 10, 20 μM of TAPI-0 or in media alone for 2 hours; and then stimulated with a combination of IL-7 (10ng/ml) and α-CD3/α-CD28 antibodies (1μg/ml). Supernatants were collected after 72 hours and the concentration of sCD127 was quantified by ELISA (n = 4). Bars represent mean ± SEM. *p=0.0001 as measured by One-way ANOVA and <0.05 by pairwise Dunnett test versus control (no inhibitor).

Fig.3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sCD127 (pg/ml)
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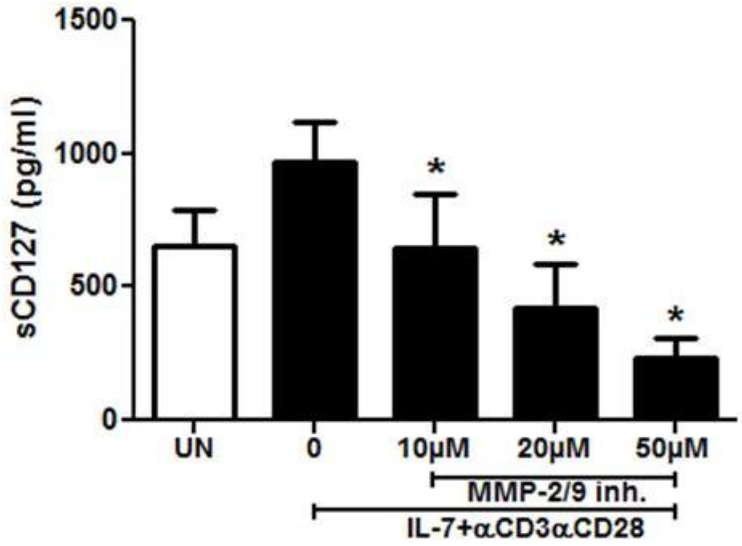
UN
0
5μM
10μM
20μM
TAPI-0
IL-7+αCD3/αCD28

Figure 4. Inhibition of MMP-2 and MMP-9 causes significant reduction in the amount of sCD127 released; while inhibition of MMP-3 has no effect on sCD127 release from $CD8^+$ T-cells.

$CD8^+$ T-cells were incubated for 2 hours with either an MMP-2/9 inhibitor or a MMP-3 inhibitor (10, 20 and 50μM) then stimulated with IL-7 (10ng/mL) plus αCD3/αCD28 (1μg/mL each). Supernatants were collected after 72 hours and the concentration of sCD127 was quantified by ELISA. **(A)** Concentration of sCD127 in culture supernatants following MMP-2 and -9 inhibition (n = 6). Bars represent mean ±SEM. * p=.0305 as measured by One-way ANOVA and $p < 0.05$ by Dunnet's test**. (B)** Concentration of sCD127 in culture supernatants following MMP-3 inhibition (n = 6). Bars represent mean ±SEM. No significant difference was measured by One-way ANOVA.

Fig.4 (A)


1500
1000
500
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sCD127 (pg/ml)
*
*
*
UN
0
10μM
20μM
50μM
MMP-2/9 inh.
IL-7+αCD3αCD28

Fig.4 (B)

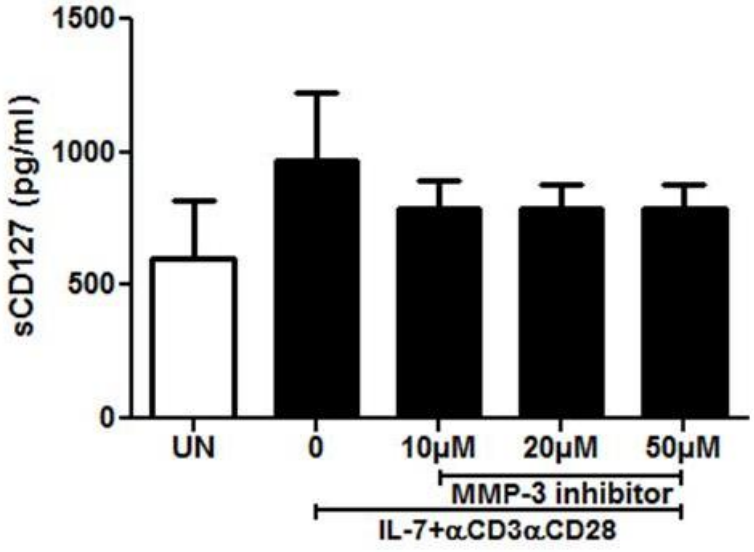

1500
1000
500
0
sCD127 (pg/ml)
UN
0
10μM
20μM
50μM
MMP-3 inhibitor
IL-7+αCD3αCD28

Figure 5. MMP-9, but not MMP-2, is involved in the release of sCD127.

$CD8^+$ T-cells were incubated for 2 hours with either MMP-2 or MMP-9 inhibitor (10, 20 and 50 µM) then stimulated with IL-7 (10ng/mL) plus α-CD3/α-CD28 antibodies (1µg/mL each). Supernatants were collected after 72 hours and the concentration of sCD127 was quantified by ELISA. **(A)** Concentration of sCD127 in culture supernatants following MMP-9 inhibition (n=4). Bars represent mean ±SEM. * p=0.0019 as measured by One-way ANOVA and <0.05 by pairwise Dunnett test versus control (no inhibitor). **(B)** Concentration of sCD127 in culture supernatants following MMP-2 inhibition (n=4). Bars represent mean ±SEM. No significant difference was measured by One-way ANOVA.

Fig.5 (A)

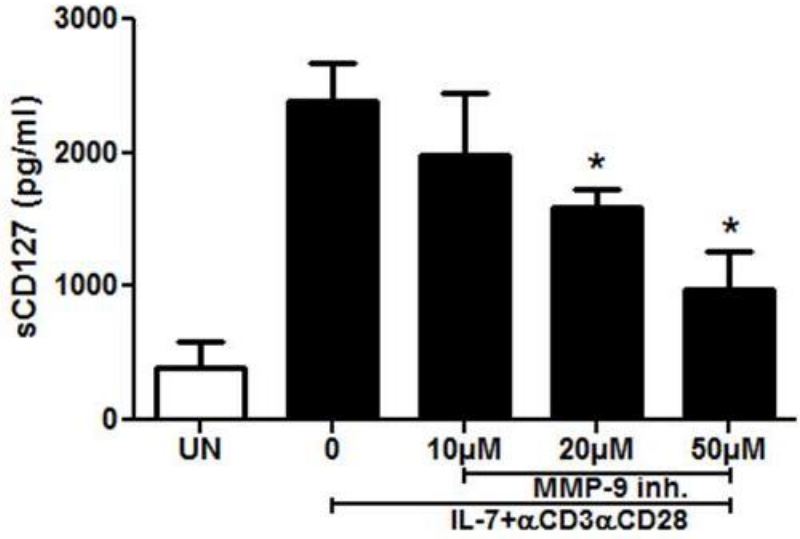
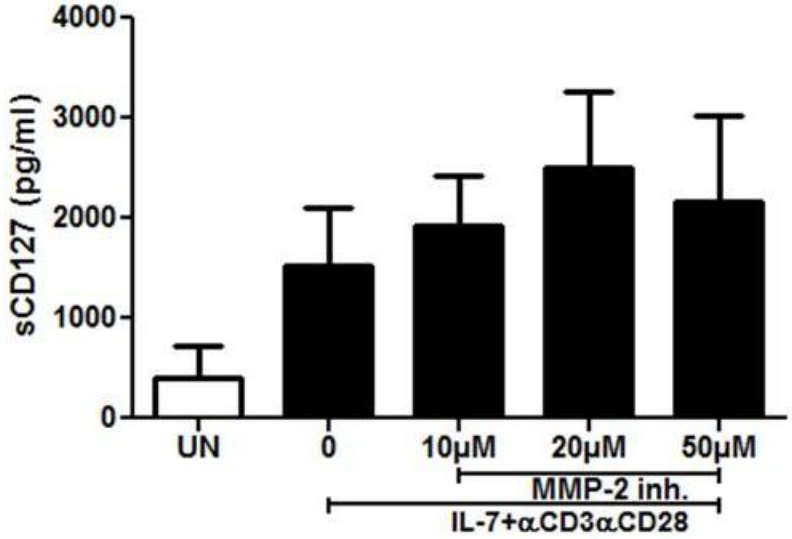

3000
2000
1000
0
sCD127 (pg/ml)
*
*
UN
0
10μM
20μM
50μM
MMP-9 inh.
IL-7+αCD3αCD28

Fig.5 (B)


4000
3000
2000
1000
0
sCD127 (pg/ml)
UN
0
10μM
20μM
50μM
MMP-2 inh.
IL-7+αCD3αCD28

3.1.1.2 The effect of cysteine and serine proteases

To determine if other proteases are involved in cleaving CD127 from the surface of $CD8^+$ T-cells, we tested the involvement of cysteine and serine proteases using specific inhibitors Leupeptin (10, 20, 50 µM) and Aprotinin (10, 20, 50 µM), respectively. Figure 6 A and B show that inhibition of these two types of proteases had no significant effect on the release of sCD127 from stimulated $CD8^+$ T-cells.

3.1.2 Level and activity of MMP-9

Thus far the data indicated a role for MMP-9 in the release of sCD127. Therefore, the levels and activity of this MMP were examined.

First, the concentration of MMP-9 in the supernatants from $CD8^+$ T-cells cultured in either media alone or stimulated with a combination of IL-7 (10 ng/mL) and αCD3/αCD28 (1µg/mL each) for 24, 48, 72 and 96 hours was quantified by ELISA. Supernatants from stimulated T-cells had significantly lower levels of MMP-9 at the time points of 72 and 96 hours (Figure 7).

Following this, the MMP-9 levels from the cell lysates of unstimulated and stimulated $CD8^+$ T-cells at the 72 hour time point were measured by ELISA. No significant difference in MMP-9 concentration was detected (Figure 8).

Figure 6. Cysteine and serine proteases are not involved in sCD127 release.

$CD8^+$ T cells were incubated for 2 hours with either Aprotinin (serine protease inhibitor; 10, 25, 50 μM) or Leupeptin (cysteine protease inhibitor; 10, 25, 50 μM) then stimulated with IL-7 (10ng/mL) plus α-CD3/α-CD28 (1μg/mL each). Supernatants were collected after 72 hours and concentration of sCD127 was quantified by ELISA. **(A)** Concentration of sCD127 in culture supernatants following cysteine protease inhibition (n=6). Bars represent mean ±SEM. **(B)** Concentration of sCD127 in culture supernatants following serine protease inhibition (n=6). Bars represent mean ±SEM.

Fig.6 (A)

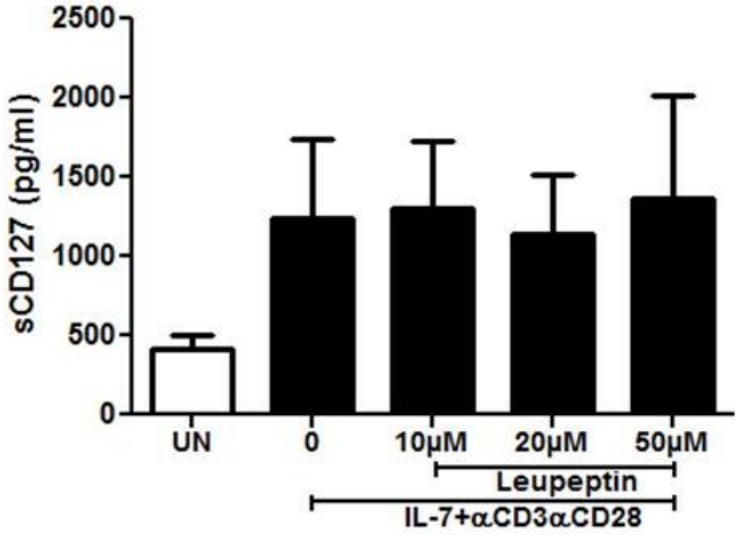

2500
2000
1500
1000
500
0
sCD127 (pg/ml)
UN
0
10μM
20μM
50μM
Leupeptin
IL-7+αCD3αCD28

Fig.6 (B)

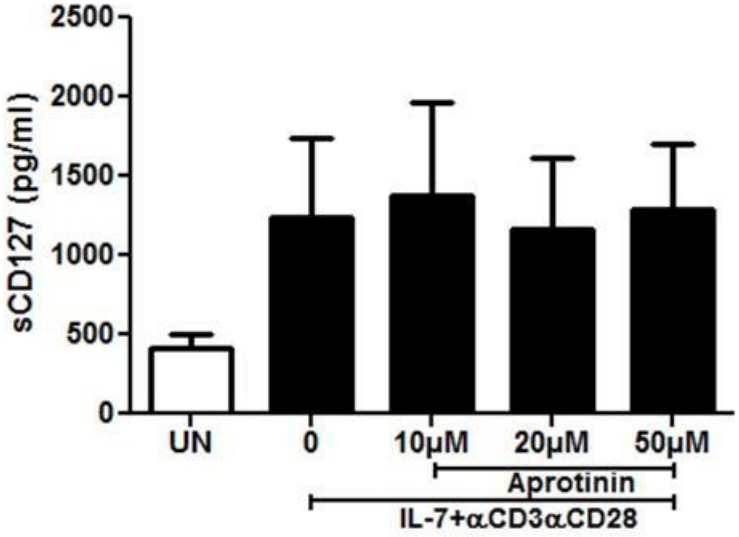

2500
2000
1500
1000
500
0
sCD127 (pg/ml)
UN
0
10μM
20μM
50μM
Aprotinin
IL-7+αCD3αCD28

Figure 7. MMP-9 levels are significantly higher in the supernatants from unstimulated $CD8^+$ T-cells compared to stimulated $CD8^+$ T-cells.

$CD8^+$ T-cells were cultured in media alone or stimulated with a combination of IL-7 (10ng/ml) plus αCD3/αCD28 (1µg/ml each). MMP-9 protein concentration in supernatants collected every 24 hours, up to 96 hours.* $p = 0.0396$ paired student t-test compared to unstimulated control.

Fig.7

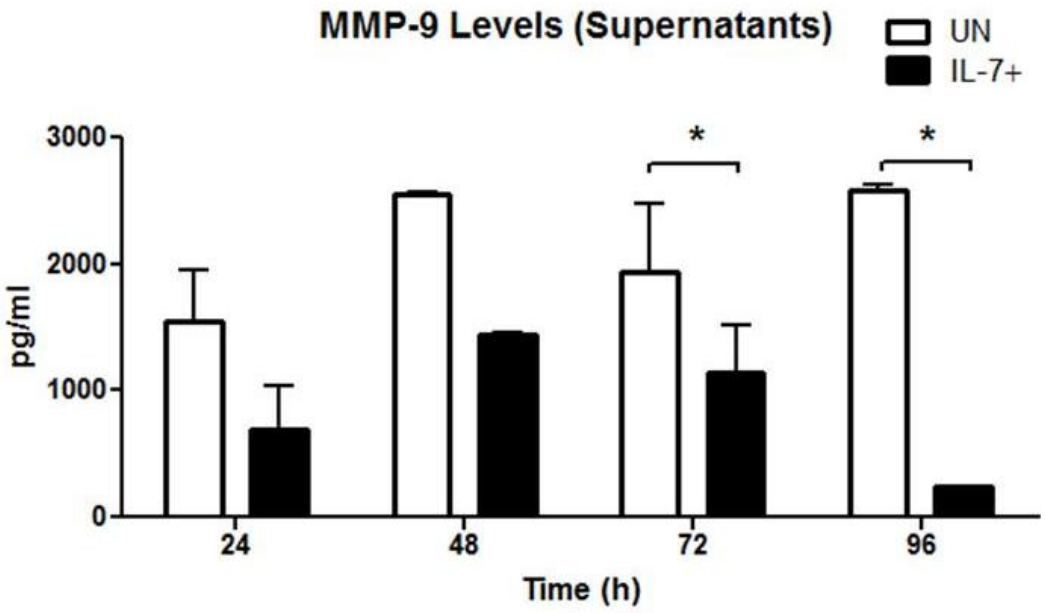
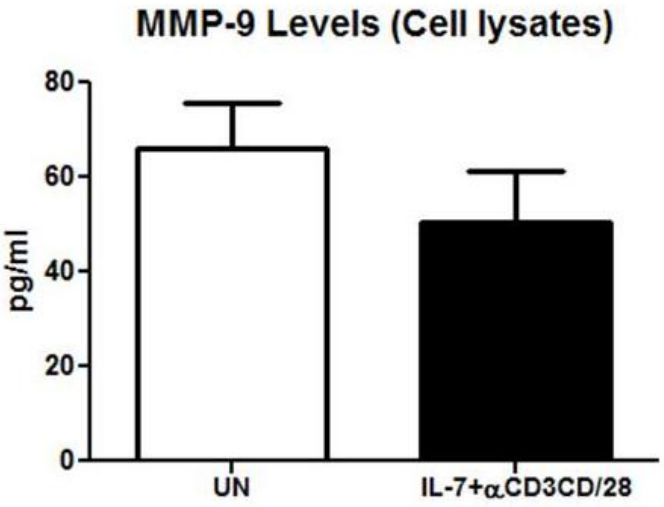

MMP-9 Levels (Supernatants)
UN
IL-7+
3000
2000
1000
0
pg/ml
*
*
24
48
72
96
Time (h)

Figure 8. No significant difference between the levels of MMP-9 in the cell lysates of stimulated and unstimulated CD8+ T-cells.

Unstimulated and IL-7 (10ng/ml) plus αCD3/αCD28 (1µg/ml each) co-stimulated T-cells were lysed 72 hours post stimulation. The concentration of MMP-9 in lysates was quantified by ELISA. Bars represent mean ± SEM (n = 4).

Fig.8



Following the quantification of MMP-9 levels in supernatants, the activity of MMPs in supernatants post IL-7 and αCD3/αCD28 stimulation was examined. The MMP activity assay is a fluorometric assay that detects fluorescence from MMP cleaved peptides. When no MMP activity is present the pro-peptide remains uncleaved and the fluorescence of one part of the pro-peptide quenches the fluorescence of the other part. In Figure 9A, a significant increase in MMP activity was detected in supernatants of $CD8^+$ T-cells cultured for 72 hours with IL-7 plus αCD3/αCD28.

MMP activity was also measured using specific inhibitors for MMP-2/-9 or MMP-3 on the stimulated T-cell cultures. This was done to determine which class of MMP was responsible for the increase of MMP activity following stimulation. As shown in Figure 9B, MMP-2/9 inhibitor significantly reduced the activity of MMPs in the supernatant of stimulated $CD8^+$ T-cells, while MMP-3 inhibitor had no significant effect on MMP activity (Figure 9C).

3.1.3 Ectodomain shedding of sCD127

To determine if this MMP dependent release of sCD127 was occurring on the surface of the cell; $CD8^+$ T-cells were labeled with biotin, incubated for 2 hours with broad inhibitors of MMPs, GM6001 and TAPI-0 (10, 20, 50 μM), and then cultured in either media or media with the combination of IL-7 (10 ng/mL) and αCD3/αCD28 (1μg/mL each) for 72 hours. Culture supernatants were collected and biotinylated proteins were recovered by affinity purification. Western blots probing for CD127 were run on isolated surface protein from supernatants. Figure 10 A and B show that sCD127 shedding into supernatants is not due to MMP activity.

Figure 9. IL-7 and αCD3/αCD28 stimulation of $CD8^+$ T-cells results in significantly elevated MMP activity compared to unstimulated cells; while MMP-2/9 inhibition results in significantly reduced MMP activity in stimulated $CD8^+$ T-cells

(A) MMP activity increases when $CD8^+$ T-cells are stimulated with IL-7 and αCD3/αCD28. $CD8^+$ T-cells were cultured in media alone or stimulated with a combination of IL-7 (10ng/ml) plus αCD3/αCD28 (1µg/ml each). Supernatants were collected after 72 hours and MMP activity was assessed by measuring fluorescence (n=7). * p= .0147 paired student t-test compared to stimulated control. **(B) MMP activity decreases when stimulated $CD8^+$ T-cells are pre-treated with MMP-2/9 inhibitor.** $CD8^+$ T-cells were cultured in the presence of specific MMP-2/9 inhibitor or media alone and then stimulated with a combination of IL-7 (10ng/ml) + αCD3/CD28 (1µg/ml). Supernatants were collected after 72 hours and MMP activity was assessed by measuring fluorescence intensity. Bars represent mean ±SEM (n=4). * p=.0029 as measured by One-way ANOVA and <0.05 by pairwise Dunnett's test versus control (no inhibitor). **(C) MMP activity is not affected after treating $CD8^+$ T-cells with MMP-3 inhibitor.** $CD8^+$ T-cells were cultured in the presence of specific MMP-3 inhibitor (10, 20, 50µM) or media alone and then stimulated with a combination of IL-7 (10ng/ml) + αCD3/CD28 (1µg/ml). Supernatants were collected after 72 hours and MMP activity was assessed by measuring fluorescence intensity. Bars represent mean ±SEM (n=4).

Fig.9 (A)

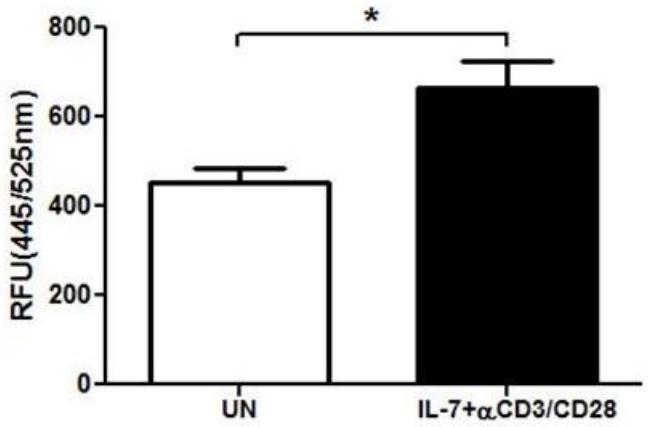


Fig.9 (B)

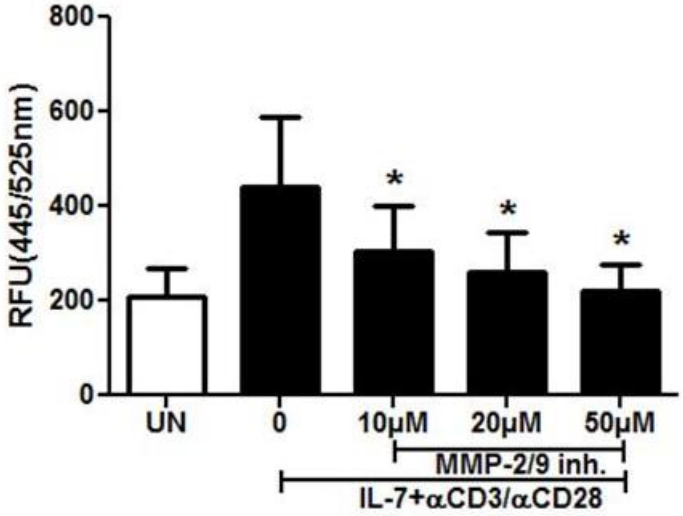


Fig.9 (C)

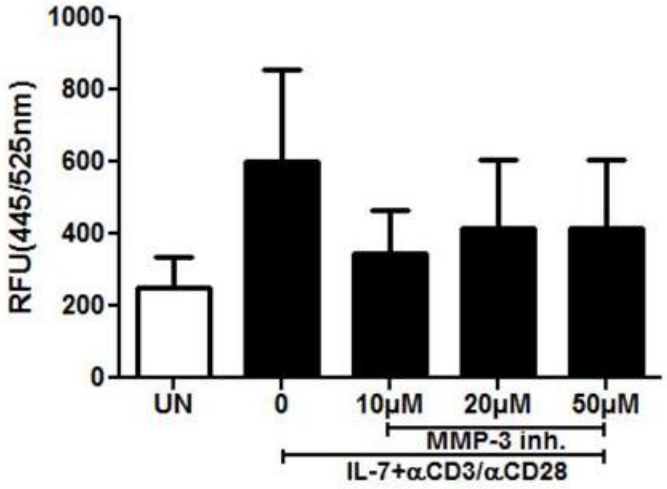


Figure 10. Ectodomain shedding of sCD127 is independent of MMP activity

(A) The broad MMP inhibitor, GM 6001, does not block ectodomain shedding of membrane CD127. (B) MMP and ADAM17 inhibitor, TAPI-0, does not block ectodomain shedding of membrane CD127. Surface biotinylated $CD8^+$ T-cells were treated with a broad MMP inhibitor, either GM 6001 or TAPI-0 (10, 20, 50 µM), for 2 hours and then stimulated with a combination of IL-7 (10 ng/ml) and αCD3/αCD28 antibodies (1 µg/ml each). Culture supernatants were collected after 72 hours and biotinylated proteins were isolated by affinity purification using a NeutrAvidin Agarose column. Samples were electrophoresed on a 10% denaturing SDS-PAGE gel, blotted onto PVDF membrane and probed with a mouse monoclonal anti-human CD127 antibody (clone R34.34). Proteins were detected by ECL chemiluminescence. (A) Representative blot of n=5. (B) Representative blot of n=3.

Fig.10 (A)

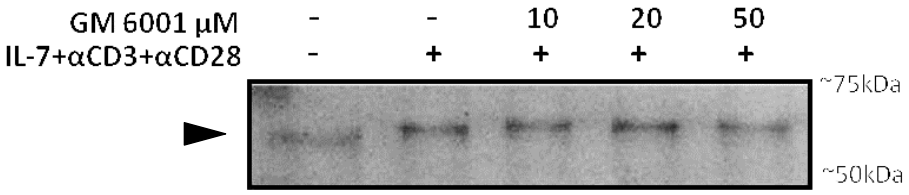
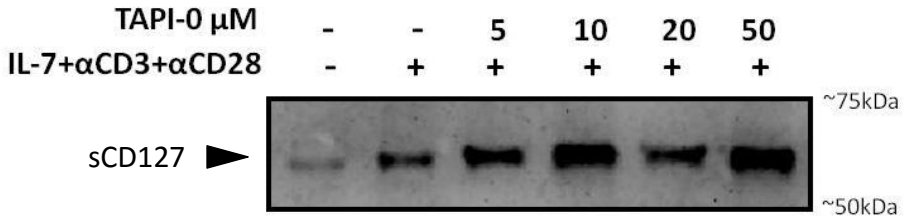


Fig.10 (B)



While the previous experiment showed that MMPs are not involved in ectodomain shedding of sCD127 from the surface of stimulated $CD8^+$ T-cells, the role of IL-7 signaling was yet to be determined in regards to ectodomain shedding. To examine the role of IL-7 signaling, STAT5, JAK, and PI3K inhibitors were used on biotin labeled $CD8^+$ T-cells that were stimulated with IL-7 and αCD3/αCD28. After 72 hours of stimulation and treatment with the inhibitors mentioned above, biotin labeled surface proteins were isolated from the supernatants and examined by Western Blot. Ectodomain shedding of sCD127 was completely blocked when $CD8^+$ T-cells were treated with 500μM of STAT5 inhibitor (Figure 11A) or 50μM of JAK inhibitor (Figure 11B), while very little sCD127 was detected when T-cells were treated with 20μM or 50μM of PI3K inhibitor (Figure 11C).

3.2 Mechanism of sCD127 release from thymocytes

3.2.1 The release of sCD127

Our lab has previously shown that human thymocytes can be supported in co-culture with a murine bone marrow cell line, OP9-DL1. To examine the release of sCD127 from human thymocytes; the release of sCD127 over time was measured in unstimulated thymocytes co-cultured with OP9-DL1 cells. In Figure 12, significant levels of sCD127 were detected in the supernatants of unstimulated thymocytes at the 72 and 96 hour time points.

Figure 11. **Inhibition of IL-7 signaling results in a decreased concentration of sCD127 in the supernatant of $CD8^+$ T-cell culture**

Biotinylated $CD8^+$ T-cells were treated with either **(A)** STAT5 inhibitor (5, 50, 500 µM), **(B)** JAK inhibitor (5, 10, 20 µM) or **(C)** PI3K (10, 20, 50 µM) inhibitor for 2 hours and then stimulated with a combination of IL-7 (10 ng/ml) plus αCD3/αCD28 (1 µg/ml each). Culture supernatants were collected after 72 hours and biotinylated proteins were isolated by affinity purification using a NeutrAvidin Agarose column. Samples were electrophoresed on a 10% denaturing SDS-PAGE gel, blotted onto PVDF membrane and probed with a mouse monoclonal anti-human CD127 antibody (clone R34.34). Proteins were detected by ECL chemiluminescence. (Representative blots of n = 4).

Fig.11 (A)

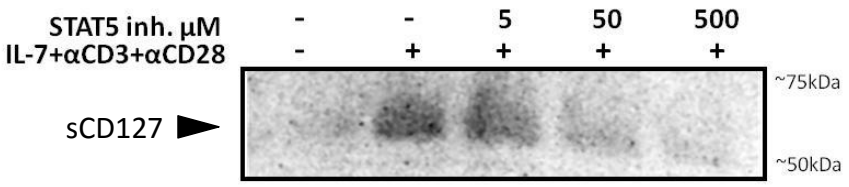


Fig.11 (B)

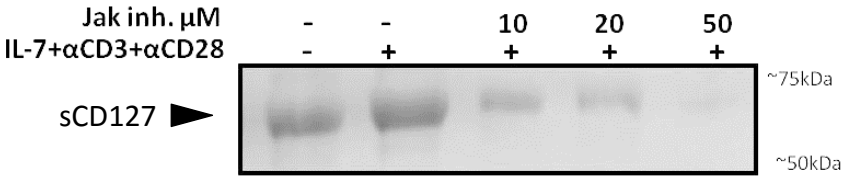


Fig.11 (C)

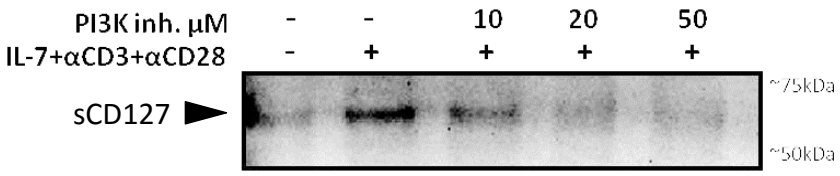
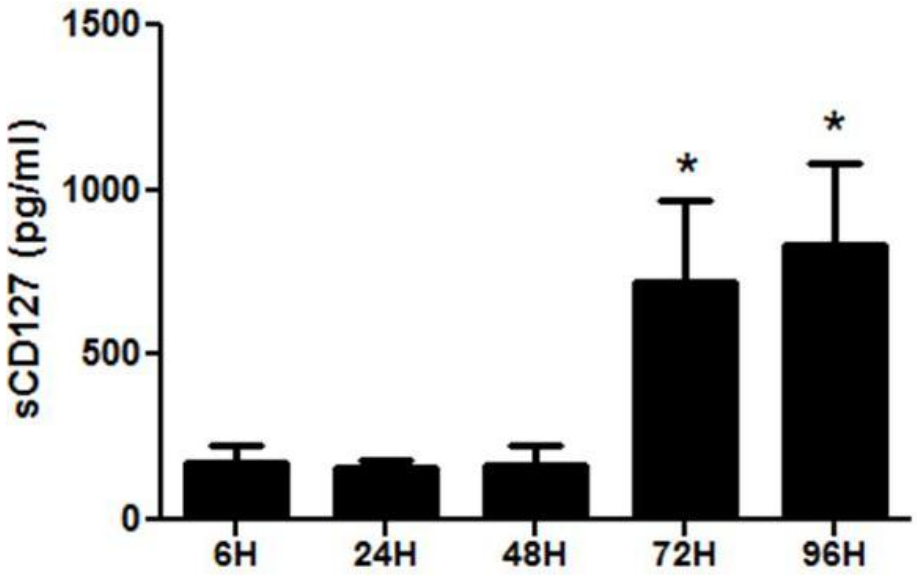


Figure 12. sCD127 is detected in the supernatants of unstimulated thymocytes over time.

Thymocytes were cultured in media alone. Supernatants were collected at 6, 24, 48, 72, 96 hours and the concentration of sCD127 was quantified by ELISA. Bars represent mean ±SEM (n = 5). * p=.0204 as measured by One-way ANOVA and <0.05 by pairwise Dunnett test.

Fig.12



To determine if stimulation of thymocytes further increases the release of sCD127, different combinations of stimulating agents were used. In Figure 13, no significant increase in the release of sCD127 was detected in the supernatants at 72 hours post stimulation with IL-7, αCD3/αCD28, PHA, αCD3/αCD28 plus IL-7, and IL-7 plus PHA when compared to unstimulated thymocytes.

3.2.2. The effect of proteolytic cleavage on sCD127 release

Next, potential proteases responsible for the release of sCD127 in unstimulated thymocytes were examined.

First, cysteine and serine proteases were examined as potential proteases mediating the release of sCD127. Figure 14A/B shows that cysteine protease inhibitor, aprotinin (10, 20, 50 μM) and serine protease inhibitor, leupeptin (10, 20, 50 μM) did not influence the level of sCD127 released by unstimulated thymocytes at 72 hours. In contrast, broad MMPs inhibitors, GM6001 (Figure 14C) and TAPI-0 (Figure 14D) prevented the release of sCD127 from unstimulated thymocytes.

After determining that MMPs were involved in the spontaneous release of sCD127 from thymocytes, the specific MMP response for this release was determined. To do so, thymocytes were treated for 2 hours with the MMP-2/9 inhibitor and MMP-3 inhibitor (10, 20, 50 μM). Figure 15A shows a significant reduction in the amount of sCD127 released into the supernatants at 72 hours with the MMP-2/-9 inhibitor. In contrast, the inhibition of MMP-3 had no effect in the amount of sCD127 released by the thymocytes (Figure 15B).

Figure 13. Stimulation of thymocytes does not significantly alter the amount of sCD127 released.

Thymocytes were cultured in media alone or stimulated with IL-7 (10 ng/ml), αCD3/αCD28 (1 μg/ml), PHA (2.5 μg/ml), IL-7 (10 ng/ml) plus αCD3/αCD28 (1 μg/ml), and PHA (2.5 μg/ml) plus αCD3/αCD28 (1 μg/ml). Supernatants were collected at 72 hours. The concentration of sCD127 was quantified by ELISA. Bars represent mean ±SEM (n = 5).

Fig.13

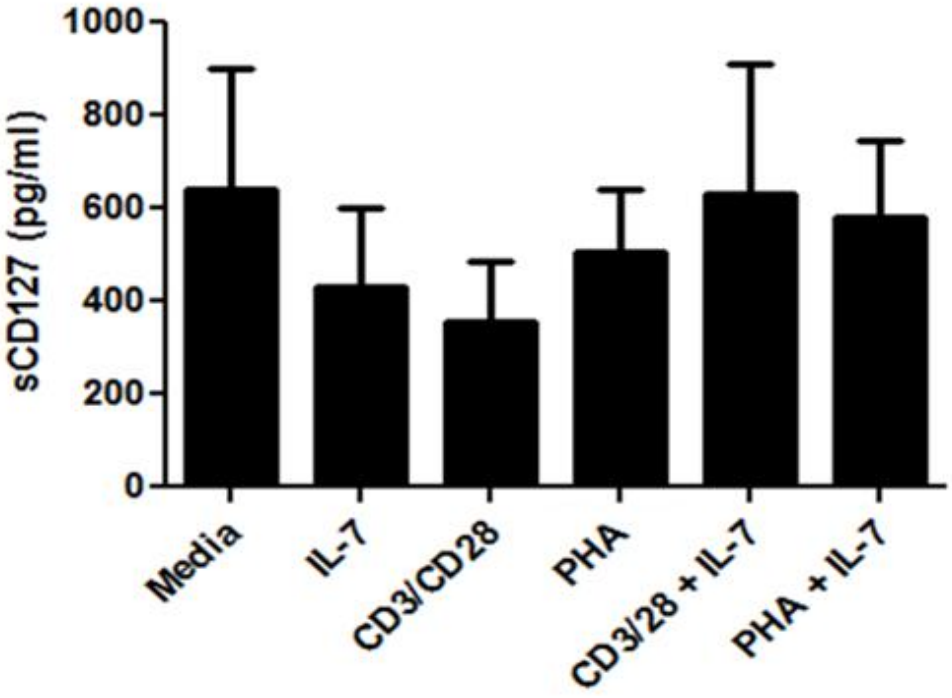

sCD127 (pg/ml)
1000
800
600
400
200
0
Media
IL-7
CD3/CD28
PHA
CD3/28 + IL-7
PHA + IL-7

Figure 14. Matrix metalloproteases, but not cysteine or serine proteases, mediate the release of sCD127 from unstimulated thymocytes.

Thymocytes were incubated for 2 hours with the serine protease inhibitor, aprotinin (10, 20 and 50 µM), the cysteine protease inhibitor, leupeptin (10, 20 and 50 µM), a broad MMP inhibitor, GM6001 (10, 20 and 50 µM), or the broad MMP and ADAM17 inhibitor, TAPI-0 (5, 10, 20 µM). Supernatants were collected after 72 hours and the concentration of sCD127 was quantified by ELISA. **(A)** Concentration of sCD127 in culture supernatants following serine protease inhibition (aprotinin). Bars represent mean ±SEM (n=5). **(B)** Concentration of sCD127 in culture supernatants following cysteine protease inhibition (leupeptin). Bars represent mean ±SEM (n=5). **(C)** Concentration of sCD127 in culture supernatants following broad MMP inhibition (GM6001). Bars represent mean ±SEM (n=6). *p = 0.0061 as measured by One-way ANOVA and <0.05 by pairwise Dunnett's test versus control (no inhibitor). **(D)** Concentration of sCD127 in culture supernatants following broad MMP and ADAM17 inhibition (TAPI-0). Bars represent mean ±SEM (n=5). *p = 0.0009 as measured by One-way ANOVA and <0.05 by pairwise Dunnett's test versus control (no inhibitor).

Fig.14

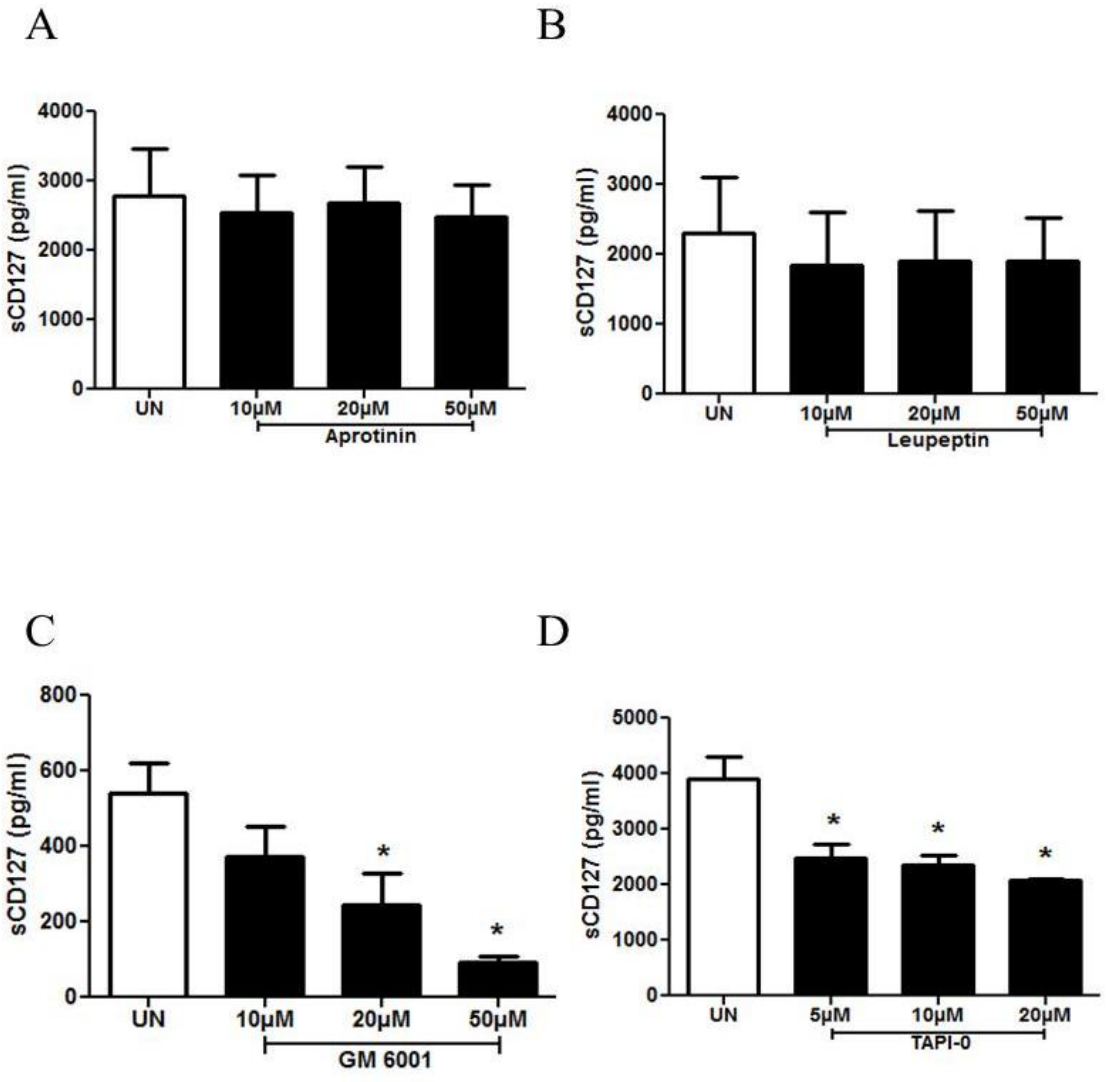

A
B
C
D
sCD127 (pg/ml)
4000
3000
2000
1000
0
UN
10μM
20μM
50μM
Aprotinin
Leupeptin
800
600
400
200
*
5000
4000
3000
2000
1000
5μM
GM 6001
TAPI-0

Figure 15. Inhibition of MMP-2/9 significantly reduces the level of sCD127 released from unstimulated thymocytes; while inhibition of MMP-3 has no effect on sCD127 release.

Thymocytes were incubated for 2 hours with no inhibitor, MMP-2/9 inhibitor (10, 20, 50 μM) or MMP-3 inhibitor (10, 20, 50 μM). Supernatants were collected after 72 hours and the concentration of sCD127 was quantified by ELISA. **(A)** Concentration of sCD127 in culture supernatants following MMP-2/-9 inhibition. Bars represent mean ± SEM (n=6). *p= 0.332 as measured by One-way ANOVA and <0.05 by pairwise Dunnett test versus control (no inhibitor). **(B)** Concentration of sCD127 in culture supernatants following MMP-3 inhibition. Bars represent mean ± SEM (n=5).

Fig.15 (A)

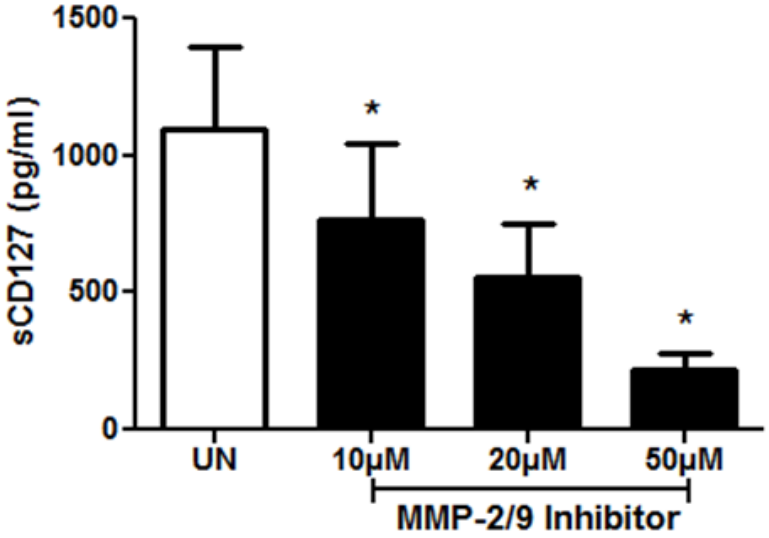
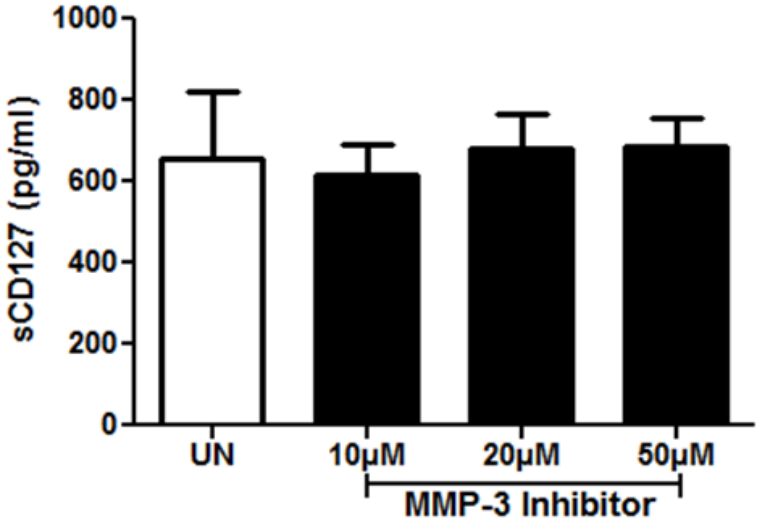


Fig.15 (B)



After determining that MMP-2 or MMP-9 was involved in the release of sCD127 from thymocytes, the level of MMP-9 and MMP-2 in the supernatants was quantified at various time points, as was previously done in $CD8^+$ T-cells. Distinct from what was found in $CD8^+$ T-cells, a significant increase in MMP-9 levels in the supernatants of cultured thymocytes was observed at the 48 and 72 hour time points (Figure 16). However MMP-2 in supernatants could not be detected using this method.

To further examine this mechanism, MMP activity from supernatants of thymocytes, treated with MMP-2/9 inhibitor and cultured in media for 72 hours, was measured using the same technique as was previously done for $CD8^+$ T-cells. As shown in Figure 17 no change in the release of sCD127 was detected.

3.2.3 Ectodomain shedding of sCD127

Finally to determine if MMPs were cleaving the ectodomain of CD127 on the surface; thymocytes labeled with biotin were treated for 2 hours with either the broad MMPs inhibitor, GM6001 (10, 20, 50 µM), or the broad MMPs and ADAM17 inhibitor, TAPI-0 (10, 20, 50 µM). After 72 hours, biotin labeled proteins were isolated from culture supernatants and probed for CD127 by Western blot. As shown in Figure 18A/B, neither of the MMP inhibitors had any effect on the shedding of CD127 ectodomain from thymocytes.

Figure 16. MMP-9 levels increase in the supernatant of unstimulated thymocytes over time.

Thymocytes were cultured in media alone for up to 72 hours. Supernatants were collected at 6, 12, 24, 48, and 72 hours and the concentration of MMP-9 was quantified by ELISA. Bars represent mean ±SEM (n = 5). *p = 0.0001 as measured by One-way ANOVA and <0.05 by pairwise Dunnett test.

Fig.16

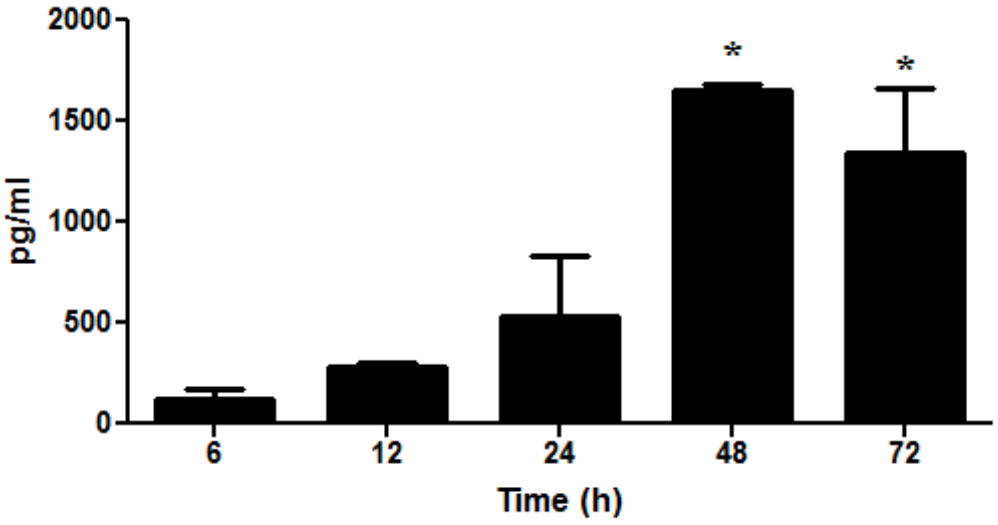


Figure 17. Activity of MMP following inhibition of MMP-2 and MMP-9.

Thymocytes were cultured in the presence of specific MMP-2 and 9 inhibitor (10, 20, 50 µM) or in media alone. Supernatants were collected after 72 hours and MMP activity was assessed by measuring relative fluorescence intensity (RFU 445/525nm) (n=4).

Fig. 17

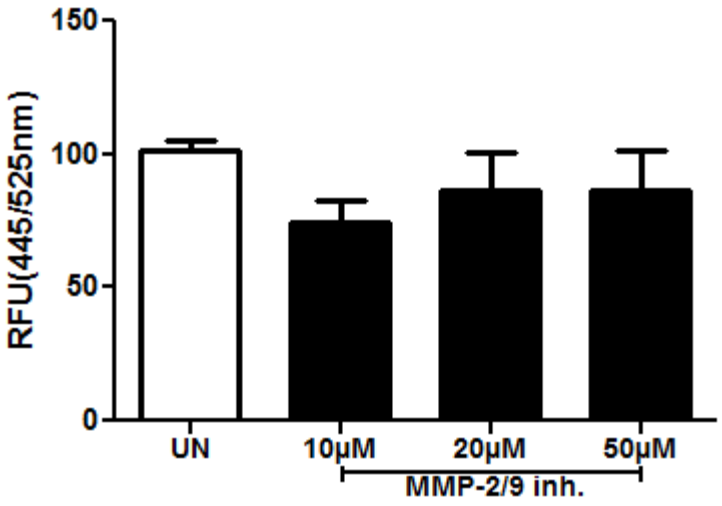


Figure 18. MMPs are not involved in ectodomain shedding of sCD127 in thymocytes.

Biotin surface labeled thymocytes were treated with either the **(A)** broad MMP inhibitor, GM6001 (10, 20, 50 µM), or the **(B)** broad MMP and ADAM17 inhibitor, TAPI-0 (10, 20, 50 µM) for 2 hours and then incubate at 37°C. Culture supernatants were collected after 72 hours and biotinylated proteins were isolated by affinity purification using a NeutrAvidin Agarose column. Samples were electrophoresed on a 10% denaturing SDS-PAGE gel, blotted onto PVDF membrane and probed with a mouse monoclonal anti-human CD127 antibody (clone R34.34). Proteins were detected by ECL chemiluminescence. (Representative blots of n = 3).

Fig. 18 (A)

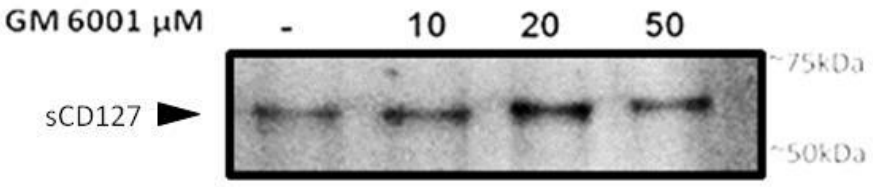
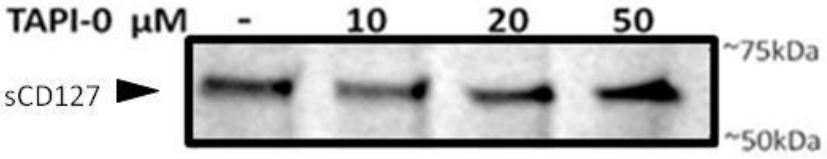


Fig. 18 (B)



CHAPTER 4: DISCUSSION

4. DISCUSSION

The tightly regulated production and complex function of soluble receptors suggests a crucial role for them in health, as well as in disease settings. Exposure to infectious agents elicits defense mechanisms that require a timely immune response, in which the relevance of soluble receptors has been widely described. Therefore it is not uncommon that soluble receptors are often used as markers of disease progression [99]. Immune function depends on a series of events that activate and amplify the reaction targeted at the antigen, and proteolytic systems are central to all of these events [55]. However, knowledge regarding the function *in vivo,* as well as the mechanism of generation for some receptors is limited; such is the case for the soluble form of the IL-7Rα. Our group and others have done extensive work to better understand the role of this receptor in health and disease scenarios [26-31].

In the last 10 years mRNA splicing has been the only mechanism described to be involved in the generation of sCD127. However, recent findings have pointed out the possibility of other mechanisms involved. We were the first group to report that ectodomain shedding was a possible alternative mechanism for the release of sCD127 in response to IL-7 [31]. Ectodomain shedding is a mechanism mainly catalyzed by proteinases of the "a disintegrin and metalloprotease" (ADAM) family and it is involved in a variety of essential functions. Cells use ectodomain shedding to actively regulate the expression and function of surface molecules, and modulate a wide variety of cellular and physiological processes.

In this study two different mechanisms of release of sCD127 were identified, one of them involves proteolytic cleavage of CD127 by MMP-9 and the second mechanism involves ectodomain shedding independent of MMPs, for which the protease responsible remains to be identified.

4.1 IL-7 dependent downregulation of CD127 receptor

IL-7 downregulates the expression of the cytokine specific receptor CD127 and various studies have looked into the mechanism behind this process. Ghazawi *et al* showed that IL-7 suppresses expression of its own receptor in $CD8^+$ T-cells by two independent mechanisms, a transcriptional one and one involving receptor internalization [100]. Through these pathways, IL-7 provides negative feedback on its signaling pathway, which results in fine-tuned immune responses in human $CD8^+$ T-cells. Furthermore, while some studies have suggested that IL-7 suppresses CD127 gene transcription, it has not been determined whether the loss of CD127 protein from the cell surface is the result of transcriptional downregulation. However, our group has shown that ectodomain shedding is a mechanism for the generation of sCD127 [31] and the work presented here further confirms this mechanism of CD127 downregulation and expression of sCD127.

4.2 Induction of sCD127 with IL-7 plus TcR stimulation

Preliminary data from our laboratory demonstrated that release of sCD127 could be induced by IL-7 plus TcR stimulation. Experiments using IL-7 alone, failed to induced the release of sCD127; which is consistent with previous studies describing how, for optimal activity, cells require further co-stimulation from either other cytokines or TcR and not just a single cytokine. Treiber-Held *et al* reported that TcR activation in combination with a cytokine can induce the release of soluble IL-2R alpha from PBMC [101]. In this study it was confirmed that release of sCD127 required co-stimulation with IL-7 plus αCD3/αCD28, as such the experimental approach used to further characterize the mechanisms involved in the release of sCD127 continued to use this co-stimulation.

4.3 Proteolytic cleavage and the release of sCD127

Proteolytic cleavage is a common mechanism of generation of soluble cytokine receptors [55-58], however it was still unclear if proteolytic cleavage of CD127 was involve in the generation of sCD127. Proteolytic processing of proteins is a common and crucial event in biology, clear evidence of this being that more than 2% of mammalian genes encode proteases [102]. Proteases have the unique ability to hydrolyze peptide bonds, irreversibly modifying the function of the substrate protein. By this specific processing, rather than the complete degradation of substrates, proteases modify signaling pathways and cell function.

In this study two distinct mechanisms were described to be involved in the release of sCD127. It has been previously described for TNF-α and L-selectin receptors, that ectodomain shedding is a prerequisite for further proteolytic processing; whether this is the case for sCD127 remains to be determined [103]. The ectodomain shedding of CD127 is most likely induced by a γ-secretase complex which is usually localized in the cell surface in a small but fully active amount. Further experiments are required to confirm this hypothesis; however using the current experimental approach it is not possible to determine whether γ-secretases are causing ectodomain shedding because there are no commercial γ-secretase inhibitors available. On the other hand it was shown here that the release of sCD127 by a clipping mechanism (Figure 1B) seems to be performed by MMP-9. It is however important to keep in mind that MMP activity is a complex network and that other MMPs may have a role in the release by either aiding in the activation of MMP-9 or enhancing its activity.

4.3.1 Role of cysteine and serine proteases in release of sCD127

The role of cysteine and serine proteases in the generation of soluble receptors and overall homeostasis of the cell environment has been well established. Despite their importance, neither cysteine nor serine proteases are involved in the endogenous release of sIL-15R [104]. Nevertheless prior to this study, there was no data about the role of these proteases on the release of sCD127. In my experiments, inhibition of serine and cysteine proteases had no effect on the release of sCD127 (Fig. 6A and B); as was similarly observed with IL-15R. This suggests that cysteine and serine proteases are not involved in the release of sCD127.

Interestingly, these proteases although not identical, do have very similar catalytic mechanisms. Serine proteases utilize their HO- side chain in the catalytic site to interact with the substrate, whereas cysteine proteases use their HS- side chain. These enzymes are folded into two relatively large globular domains surrounding a cleft that contains the catalytic site; as such substrates must enter the cleft in order for cleavage to occur [105]. Considering the data suggests serine and cysteine proteases do not cleave CD127, I propose that the CD127 structure does not fit into the cleft of these proteases, therefore CD127 does not bind to the catalytic site and proteolytic cleavage is not achieved. This theory can be confirmed with further crystallographic analysis of the CD127 receptor in conjunction with the proteases.

4.3.2 MMPs in the release of sCD127

In recent years the initial idea of MMPs as just enzymes involved in the degradation of extracellular matrix has evolved into defining them as key processing enzymes that can modulate cell behavior through precise and efficient cleavage of bioactive molecules,

including cytokines and cytokines receptors. Following recent evidence linking MMPs to several different roles within the cell, the role of these enzymes in the potential release of sCD127 receptor was studied.

Following incubation with GM6001, a broad MMP inhibitor, a dose dependent decrease on the release of sCD127 was measured (Fig.2). These data suggests a role for MMPs in the cleavage of CD127. To confirm these results and to further characterize the specific proteases involved in this release, specific inhibitors for MMP-2/-9 and MMP-3 were used. These MMPs have been widely described as being involved in the cleavage of several cytokine receptors and other molecules. Interestingly, MMP-2/-9 inhibition had a dose dependent effect in the release of sCD127 (Fig. 4A), whereas no change in the release was measured when using the MMP-3 specific inhibitor (Fig. 4B).

MMP-2 and MMP-9 had been previously reported to cleave other cytokine receptors however this is the first time CD127 has been described as a substrate for these MMPs. MMP-2 and -9 are both gelatinases that have been shown to cleave basement membrane type IV and V collagen, elastin and gelatins (denatured collagens) therefore they are known as gelatinase A and B respectively [56, 69]. Despite both being able to cleave gelatinases, the substrate specificity is not identical. In addition, MMP-9, and to a lesser extent MMP-2, can cleave a variety of non-ECM components including IL-1β. To determine if one or both gelatinases had an effect on the release of sCD127, specific inhibitors for either MMP-9 or MMP-2 were used. Inhibition with MMP-9 inhibitor showed a dose-dependent decrease in the release of sCD127 (Fig. 5A), whereas following MMP-2 inhibition there was no change in the release of this soluble receptor (Fig. 5B). Taken together, these data show that MMP-9 is involved in the release of sCD127 in response to IL-7 plus TcR stimulation. It was

interesting to find a clear distinction in activity between the two gelatinases, because although they are close structural homologues, MMP-2 does not appear to be activated by the same mechanism.

To confirm the previous data, as well as determine the involvement of ADAMs, in particular ADAM17. TAPI-0, a broad MMP inhibitor which is design to inhibit the TNF-α converting enzyme (ADAM 17) as well as other MMPs was used. A significant decrease on the release of sCD127 was measured even at the lowest concentration (5μM) of TAPI-0 (Fig. 3). Our previous experiments showed the role of MMPs in sCD127 release, however to be able to determine if ADAM 17 is also involved in this release, a specific inhibitor that can exclusively inhibit the ADAMs family activity without affecting MMPs activity is required, however such inhibitor is yet to be available.

4.3.2.1 MMP Levels on $CD8^+$ T-cells

In order to have a better understanding of the mechanisms leading to the release of sCD127, concentrations of different MMPs (MMP-2, -3 and -9) in culture supernatants were measured. Endogenous proteins levels were compared to levels following IL-7 plus TcR stimulation. Only MMP-9 levels in culture supernatants could be detected with the specific ELISA kit used. This may have been due to the sensitivity of the assay for the MMP-2 and MMP-3 ELISA.

Furthermore, MMP-9 protein levels in culture supernatants from untreated cells were higher than supernatants from cells stimulated with IL-7 plus antiCD3/CD28 (Fig. 7). A potential explanation for this is that MMP-9 is being recruited by the cell for the cleavage of CD127, or other activities, thereby limiting its availability in culture and suggesting that

MMP-9 is being consumed. To further characterize this potential mechanism, MMP-9 protein levels from cell lysates were also measured. Protein levels from untreated cells were not different when compared to IL-7/TcR stimulated cells; which may be the result of MMP-9 being recruited to the cell surface for activation by other protease families including other MMPs (Fig. 8) However, assessment of pro-MMP-9 by ELISA alone is limited by the ability of this technique to clearly distinguish between the fully active MMP-9 and the intermediate inactive form.

4.3.2.2 MMP activity in the release of sCD127

To have a better understanding of the effect of IL-7 plus TcR stimulation on MMP activity, the broad activity of MMPs from unstimulated cells was compared to that of cells treated with IL-7 plus TcR. Following IL-7/TcR stimulation, a significant increase in activity of MMPs in culture supernatants was found (Fig. 9A). This result may be due to increased MMP-9 recruitment for cleavage upon stimulation. Unlike MMP-2, which is mostly constitutively expressed, MMP-9 has a restricted pattern of expression. MMP-9 is highly regulated by many cytokines and growth factors. The activity of MMPs significantly decreased following the use of specific inhibitors for MMP-2 and MMP-9, (Fig. 9B) but no significant change in MMP activity was measured following MMP-3 inhibition (Fig. 9C). Taken together, this data suggests a strong correlation between MMP-9 activity and the release of sCD127. This is the first time CD127 is identified as a substrate for MMPs and in particular MMP-9. Further studies will be required to determine where in the cell MMP-9 is being recruited to, whether at the surface level by a mechanism similar to regulated intramembrane proteolysis or inside the cell.

4.4 Ectodomain shedding of the CD127 receptor

To further characterize the specific mechanism(s) leading to the release of sCD127, a biotinylation assay was performed. The objective of this assay was to determine if the receptor was being released directly from the surface by a mechanism defined as shedding. This particular assay allows one to distinguish sCD127 being released as a result of ectodomain shedding and sCD127 generated by other mechanisms. This assay consists of coating the surface of $CD8^+$ T-cells with biotin prior to the stimulation with IL-7 plus TcR, and ensures that all surface proteins, including CD127, would be labelled with biotin. Following the same experimental design previously described, after 72 hour of incubation with stimulants culture supernatants were collected and the target protein was recovered by affinity purification. This allowed the recovery of only proteins labeled with biotin and the removal of all unbiotinylated proteins. The recovered proteins were probed for CD127 using Western Blot. Through this method, only CD127 proteins that where shed from the surface would appear in the blot, while all other proteins remained excluded from the loading sample. As demonstrated in Figure 11A, IL-7 plus antiCD3/CD28 stimulation resulted in sCD127 shedding.

4.4.1 IL-7 signaling and sCD127 ectodomain shedding

Previous studies in our lab have demonstrated the role of IL-7 signaling in the release of sCD127; when $CD8^+$ T-cells where treated with either Jak, STAT5 or PI3K inhibitor, the recovery of CD127 expression was measured. After investigating the mechanisms involved in the release of sCD127, the role of IL-7 signaling was determined. To address the role of the Jak, STAT5 and PI3K signaling pathways, cells were treated for 2 hours with specific pharmacological inhibitors, followed by IL-7 plus TcR stimulation and the concentrations of

sCD127 were measured by ELISA. Inhibition of the Jak-STAT5 signaling pathway had a significant effect in the release of sCD127, which coincide with downregulation of CD127 receptor expression upon stimulation with IL-7. These results suggest an additional mechanism responsible for the downregulation of CD127 to what has been previously described. Furthermore the PI3K signaling pathway was also shown to be involved in the release of sCD127, which further confirms the involvement of the IL-7 signaling pathway in this release.

To characterize the potential role of IL-7 signaling in CD127 ectodomain shedding, pharmacological inhibitors for Jak, STAT5 and the PI3K pathway were used. A dose-dependent decrease in the amount of sCD127 being shed from the surface was detected by western blot analysis (Fig. 11 A, B, C), with similar results being found amongst the three inhibitors used. Taken together these data indicates that shedding of CD127 is an IL-7 signaling dependent mechanism.

4.4.2 MMPs and ectodomain shedding

After determining the role of IL-7 in sCD127 shedding, the specific protease(s) responsible for this process remained to be identified. Forsyth *et al*, reported that proteases involved in the shedding are most likely distinct from matrix-type metalloproteinases [106]. However, no data is available regarding MMPs and CD127 shedding. To address this question, a MMP broad inhibitor (GM 6001) was again used. Interestingly, GM6001 did not block the shedding of sCD127, even at the highest concentration used (20μM) (Fig. 10A). This was interesting finding since it revealed a novel mechanism, different from the one previously identified in this study, which is dependent on IL-7 signaling but MMP

independent. A similar scenario where two independent mechanisms contribute to the release of a receptor has been reported for Notch ligand Delta-like 1 which undergoes a sequence of shedding and regulated intramembrane proteolysis by ADAMS and γ-secretases in a signal-independent manner [107].

Finally, the role of ADAM 17 in sCD127 release was investigated. This member of the ADAMs family has been reported to have a role in the release of other receptors. No change in the release of sCD127 by ectodomain shedding was measured after using a combined TNF- processing inhibitor and MMP inhibitor known as TAPI-0, (Fig. 10B). Further studies to determine the specific protease responsible for this ectodomain release will be necessary.

4.5 The release of sCD127 release from human thymocytes

The distribution of thymocytes within the human thymus has been widely studied [108]. The majority of thymocytes isolated from thymic tissues are double positive (DP) cells, which are at a maturation stage where they undergo massive expansion to optimize the number of cells that are undergoing negative selection [108]. The expression profile of CD127 on thymocytes corresponds to the functional requirements of each subset for IL-7 signaling. Initially progenitor cells require IL-7 for survival and expansion which is reflected in the expression of CD127 on triple negative (TN) and immature intermediate single positive (ISP4) subsets. The DP subsets, that are undergoing massive apoptosis due to negative selection, down modulate CD127 on their cell surface, possibly to attenuate the survival signals provided by IL-7. Human DP thymocytes express low levels of CD127 and in mice CD127, expression on the DP subsets is completely lost presumably to avoid IL-7 survival signals and the initiation of self-reactive immune cells [109, 110].

4.5.1 Thymocytes and OP9-DL1 co-culture

T-cell development requires a specialized microenvironment that is mainly provided by the thymic stroma [111]. A number of culture systems have been developed to study T-cell development *in vitro*. For this study we used an OP9-DL1 culture system which has been widely used for the study of commitment of precursor T-cells and subsequent development of cells of T-cell lineage [32]. Despite the fact that this is a mouse stromal cell line, it has been shown to support the development of human hematopoietic stem cells and human cord blood in long term cultures [112, 113]. The importance of a co-culture system relies on the fact that thymocytes cultured alone exhibit significantly reduced viability and respond poorly to IL-7. Co-culture with OP9-DL1, cells protects thymocytes from apoptosis. Notch signaling leads to protection from T-cell receptor (TCR) induced apoptosis, hence thymocytes co-cultured with OP9-DL1 which express notch, are expected to have reduced apoptosis [114-117].

4.5.2 Endogenous release of sCD127 from human thymocytes

To characterize the release of sCD127, a panel of different combinations of stimuli similar to the one used for $CD8^+$ T-cells were used. This panel included IL-7, IL-7 in combination with PHA, IL-7 in combination with αCD3 and αCD28, as well as each antibody on its own. None of these combinations were able to induce the release of sCD127 across the different time-points examined (6, 12, 24, 48, 72 and 96 hours) (Fig. 13) which was in contrast to the results obtained with $CD8^+$ T-cells. However an increase of sCD127 over time was detected from thymocytes cultured in just media; the level of sCD127 released was significantly higher after the 72 hour time point (Fig. 12).

4.5.3 Proteolytic cleavage of CD127 in thymocytes

To determine if proteolytic cleavage was one of the mechanisms responsible for the release of sCD127, a panel of specific pharmacological inhibitors were used. This panel consisted of a cysteine and serine protease inhibitor, leupeptin and aprotinin respectively, as well as a broad MMP inhibitor (GM6001). No decrease in the release of sCD127 was observed when either leupeptin or aprotinin was used (Fig. 14 A, B). However, a dose dependent decrease in the amount of sCD127 following MMP inhibition was detected (Fig. 14 C, D). Interestingly, the importance of MMPs in the development of T lymphocytes has previously been described. Studies using explanted fetal thymus organ cultures (FTOC) have shown that T-cell development was inhibited beginning at the DN thymocyte stage when inhibitors of metalloproteinases were used in culture with thymocytes [118].

To further characterize the specific MMPs involved in this release, specific MMP inhibitors were used. Each inhibitor was chosen following previous studies that had reported MMPs relevant to the thymic microenvironment, as is the case of MMP-2 and MMP-9. Evidence has shown that thymic cells produce MMP-9, which is considered the largest and most complex member of the MMPs family [118]. Interestingly, a decrease on the release of the soluble receptor when using the MMP-2 and MMP-9 gelatinases inhibitors was measured (Fig. 15A). No effect was detected in the sCD127 protein levels following MMP-3 inhibition (Fig. 15B). These data suggest a role for gelatinases in the endogenous release of sCD127 and this is the first time CD127 has been described as a substrate for MMPs in human thymocytes.

4.5.3.1 MMP activity on thymocytes

To complete the results from the MMP inhibition experiment, endogenous MMP levels where measured. Possibly due to a lower sensitivity of the Human ELISA Kit only MMP-9 was detected. The concentration of MMP-9 increased over time, and after the 72 hour time point there was a significant increase in protein levels (Fig. 16). This increase in MMP-9 levels coincided with the increase in sCD127 levels which may be an indication of the involvement of MMP-9 in the release of sCD127. Furthermore, when measuring MMP activity following the use of specific inhibitors for MMP-2 and MMP-9, no statistical significant decrease in activity was seen (Fig. 17), though this might have been a result of the small number of experiments performed. To get a better understanding of the activity of MMPs on thymocytes further experiments will be required.

4.5.4 Ectodomain shedding of sCD127 in thymocytes

Ectodomain shedding is one type of proteolytic cleavage and therefore its potential involvement in the release of sCD127 was examined here and whether MMPs had a role in this process. A previous study from our group had shown shedding of CD127 from $CD8^+$ T-cells [31]. The use of a biotinylation assay allows recovery of surface molecules being shed by cells. This assay revealed that CD127 was being constitutively shed from the surface of thymocytes, which was an interesting finding since shedding of receptors in thymocytes has only been shown in mouse models [119] thus is a novel mechanism of CD127 regulation.

After identifying one of the mechanisms of sCD127 release, experiments to determine the protease(s) responsible for the shedding of CD127 were performed. Shedding of the receptor was not blocked following broad inhibition of MMPs (Fig. 18). This was an

interesting finding since we would have expected MMPs to be involved in this mechanism. However similar results were reported in a study of CD100 receptor in Jurkat T-cells, were CD100 shedding was not altered by MMP inhibitor GM 6001 [120]. Their results as well as the data presented in this study suggest that these receptors are released via a particular proteolytic pathway different from those described here.

To further confirm these results and to identify the specific protease possibly responsible for the release of sCD127, a TNF-α processing inhibitor (TAPI-0) was used; TAPI-0 broadly inhibits MMPs as well as ADAM 17 (also known as TACE). However no change in the release of sCD127 was detected, even at the highest concentration of the inhibitor (20μM). This suggests that MMPs and ADAM 17 are not involved in the release of sCD127 in thymocytes.

CHAPTER 5: CONCLUSION

5. CONCLUSION

Interleukin-7 has a crucial role in the development, function and survival of T-cells. The significance of IL-7R regulation has been widely described and attributed to several different biological roles. A selective role has been proposed for the upregulation of IL-7R on memory cells; T-cells expressing the highest levels of CD127 would receive the most survival signals and therefore persist. On the other hand, downregulation occurs after T-cells are exposed to γc cytokines as an altruistic mechanism to allow other cells that have not been exposed to IL-7 to receive the survival signal [34]. It has been proposed that sCD127 diminishes excessive IL-7 consumption and, thus enhances the bioactivity of IL-7 to ensure that all T-cells get the required signals. Given the importance of IL-7 in different aspects of T-cell maintenance, it is common for different infections to be characterized by the dysregulation of IL-7 receptor expression; such is the case for HIV and hepatitis C as well as other inflammatory autoimmune diseases such as multiple sclerosis and rheumatoid arthritis. A better understanding of the relation between IL-7 and its receptor is crucial to develop novel therapeutic strategies.

In this study, two different mechanisms were identified to be involved in the generation of sCD127, one of them is dependent on MMP activity whereas the other is not. These mechanisms where elucidated after examining the potential role of several proteases including cysteine, serine and metalloproteases (MMPs and ADAM 17). After determining that MMPs were involved in the release of sCD127, we sought to determine the particular MMP class involved. The data from this study show MMP-9 to have a crucial role in the release of sCD127.

To further characterize this release, the specific mechanism of generation was determined using a biotinylation assay. This assay revealed a second MMP independent mechanism occurring on the surface both $CD8^+$ T cells and thymocytes. Ectodomain shedding converts membrane-associated proteins into soluble receptors and, at the same time, rapidly reduces the level of cell surface expression. The TNF-α converting enzyme (TACE), a disintegrin and metalloprotease 17 (ADAM17), is the most well described proteases, but other proteinases can also induce the release of protein ectodomains such is the case of γ-secretases, as well as MMPs. Nevertheless, while trying to characterize the specific proteases involved in the release of sCD127, MMPs and potentially ADAM 17 were excluded as being involved in the shedding of the receptor. Future experiments must therefore be performed to characterize this specific protease involved.

For many years, studies on proteases focused on their original roles as efficient enzymes associated with protein degradation. However, the realization that, beyond these nonspecific degradation functions, proteases catalyze highly specific reactions of proteolytic processing, producing new protein products, and has led to a new era in protease research. Proteases are involved in many important cellular systems having different roles, therefore the development of protease inhibitors to target different diseases such as heart and renal failure, cancer, stroke, Alzheimer and many more is an exciting and fast growing field.

Matrix metalloprotease inhibitor development generated a lot of attention at first, because it represented the promise of a treatment that could stop different types of cancers from reaching advanced states of metastasis. The initial assumption was that by targeting MMPs, metastasis could be prevented and cancer would become a more treatable disease. However the excitement led to a failed path, when different inhibitors were rushed in to

clinical trials before the effects of them were fully understood. Clinical trials started with only three MMPs been identified; while, to date, there are about 23 known MMPs associated with different cell types. Further studies showed that MMPs are involved in a number of important normal processes, such as cell-surface-receptor cleavage and release, and cytokine and chemokine activation and inactivation. Inhibiting MMPs could interfere with normal tissue function, causing unknown side effects and possibly inhibiting useful host defense processes that control cancer growth. What once looked simple is now recognized to be a very complex, interdependent system, with the activity of many MMPs being regulated at many different levels, and each MMP being produced and activated in multiple steps and then controlled by endogenous inhibitors.

Taken together, these findings may help better understand this complex and fascinating receptor regulation system. Identifying a connection between IL-7 receptor and MMPs is an exciting finding due to the potential clinical implications. Despite the fact that most studies are focused on the role of MMPs and tumor invasion, recent studies have pointed out the relevance of these proteases in other diseases such as arthritis, hypertension, heart failure and diabetes. Despite the clear need for more work on selective inhibitors, it is clear that broad-spectrum MMP inhibitors will be needed to treat cancer at particular stages. Six MMP inhibitors are currently in advanced stages of clinical development. However a better understanding of MMP activity in different physiological and pathological processes is still needed. Understanding the detailed mechanisms by which IL-7 receptor is regulated may well lead to novel therapeutic strategies aimed at modulating T-cell numbers and function in other diseases in which cellular immune function and IL-7 activity are altered.

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APPENDIX

I. Ethics approval and consent form from the Ottawa Hospital Research Ethics Board

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Information Sheet and Consent Form for Blood and/or Semen Collection and/or Colon Biopsies

Study: Impaired Immune Function during HIV Infection

Funding Agency: Canadian Institutes of Health Research

Principal Investigator: Dr. Paul MacPherson
613-737-8899 ext 73896

Introduction:
You are being asked to participate in a study focused on immune function in HIV infection. Before you decide to participate, it is important that you understand why this research is being conducted. Please take a moment to read the following information, and ask questions if there is anything that you find unclear.

Background:
HIV infection over time causes severe impairment of the immune system. HIV enters some immune cells such as CD4 T-cells and monocytes and either kills them or prevents them from functioning properly. Other immune cells such as CD8 T-cells are not infected by HIV but are still unable to function in the presence of HIV. By limiting the activity of these various immune cells, HIV weakens the entire immune system. When the immune system is weak, HIV is able to replicate freely and eventually people can become sick from opportunistic infections.
How HIV inactivates these different cells is not yet understood.

HIV infected cells travel through the blood to many sites in the body including the testicles and prostate. Once in these tissues, HIV may behave differently than when it is present in the blood or lymph nodes. Sometimes HIV can be detected in the semen even when it is undetectable in the blood. Control of HIV replication in the semen then may not perfectly mirror what occurs in the blood.

HIV can also infect the cells that line the colon, a part of the digestive tract, and the effect it has on the immune system in the gut may also not mirror what occurs in the blood or semen.

Purpose:
One of the goals of this research program is to determine how the activities of various immune cells change during HIV infection. This will be done by examining changes in the expression of molecules on the cell surface as well as changes in overall cell function. We will compare these changes between HIV negative individuals, HIV+ patients with active viral replication, HIV+ patients on antiretroviral therapy, and in long term nonprogressors.

Another goal of this research program is to determine how immunologic control of HIV differs between different tissue compartments, namely the blood, semen, and the digestive tract. This will be done by examining HIV viral loads and levels of immune modulators isolated from semen

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or gut. Cells of the immune system involved in controlling HIV replication may also be isolated from semen or gut in order to examine their functional abilities.

Study Procedures:
Participants will be typically asked to provide a sample of either blood **or** semen **or** gut tissue. Participants who provide semen or gut samples may be also asked to provide blood.

Blood Samples:
Participants will be asked to donate samples of blood ranging from 10 ml, less than 1 tablespoon ~~(one tube) to a maximum of 200ml, approximately 14 tablespoon (less than one half the volume~~ drawn by Canadian Blood Services for routine blood donation; one unit = 500ml, approximately 33 tablespoon). White cells will be isolated from the blood and analyzed in the laboratory for changes in the expression of different molecules, and for immune activity. Serum may also be examined for molecules which regulate the activity of the immune cells.

Semen Samples:
Participants may be asked to donate semen samples. Sterile containers will be provided. Samples will be collected privately by the participant but must be delivered to the clinic within two hours.

Gut Samples:
During your colonoscopy procedure small biopsies of the colon wall will be performed in order to gather gut-associated lymphoid tissue. Approximately 30 tissue biopsies will be obtained, each consisting of about 0.3mm of tissue (9 mm total).

Duration:
Your participation will be complete once the blood and/or semen and/or gut tissue sample is obtained.

Risks:

Blood Samples:
The risks of participating in this study are those associated with routine blood drawing and may include minor pain at the site where blood is drawn and minimal bruising. Some people may experience temporary lightheadedness after drawing blood. If this occurs, you may be asked to rest in the clinic for a short period of time until the lightheadedness passes.

Semen Samples:
There are no risks associated with semen donation. Semen samples will only be used to determine HIV viral loads and for immunologic studies. Under no circumstances will the semen be available to achieve fertilization under any conditions.

Gut Samples:
During the procedure, you may feel pressure in the rectum similar to the sensation you feel with the urge to have a bowel movement. You may also feel a small amount of cramping in your abdomen as well. The most serious risk of the procedure is perforation, poking a hole in the

lining of the intestine, but it is extremely uncommon, occurring once out of every 10,000 procedures. If this occurs the attending physician will be responsible for treatment. Some bleeding will likely occur at the point where the biopsies were done. If you should experience severe discomfort during colon mucosa biopsy the procedure will be stopped immediately; you will be seen by the physician and the research nurse will monitor you until you are ready to go home.

Due to the possible bleeding following the procedure, the risk of transmitting HIV after the biopsy procedure may be increased.

Benefits:
Participation in this study will provide no direct benefits to you. It will, however, help to advance our understanding of how HIV inactivates the immune system and may lead to new strategies for the development of immune based therapies to control HIV replication.

Voluntary Participation and Withdrawal:
You are under no obligation to participate in this study. If you choose to participate, you may change your mind at any time without providing a reason. You should inform the study doctor/study staff if you decide to withdraw so that your sample may be destroyed. You are not waiving your legal rights by agreeing to take part. Whether you choose to participate or not will have absolutely no effect on the medical care you receive, now or in the future.

Confidentiality:
All personal health information will be kept confidential, unless release is required by law. Representatives of the Ottawa Hospital Research Ethics Board, as well as the Ottawa Hospital Research Institute, may review your original medical records under the supervision of Dr. MacPherson's staff for audit purposes.

You will not be identifiable in any publications or presentations resulting from this study. No identifying information will leave the Ottawa Hospital.

The link between your name and the independent study number will only be accessible by Dr. MacPherson and/or his staff. The link and study files will be stored separately and securely. Both files will be kept for a period of 15 years after the study has been completed. All paper records will be stored in a locked file and/or office. All electronic records will be stored and protected by a user password, again only accessible by Dr. MacPherson and/or his staff. At the end of the retention period, all paper records will be disposed of in confidential waste or shredded, and all electronic records will be deleted.

Your chart will be used only to obtain CD4 counts, viral loads, and overall health status. Data will be compiled using a code. Your name will not be used and will not be known to laboratory personnel. The results of this study may be presented at meetings or in publications. Your identity will not be disclosed at any time in any of these presentations or publications.

Questions About The Study:

If during the course of this study you have questions concerning the study, you may contact the principle investigator Dr Paul MacPherson at 613-737-8899 ext 73896.

The Ottawa Hospital Research Ethics Board has approved this study. This committee considers the ethical aspects of all research projects involving people. If you have any questions about your rights with regard to participating in a research study, you may contact the Chair of the Ottawa Hospital Research Ethics Board at 613-798-5555, extension 14902. Do not sign the consent form unless you have had a chance to ask questions and have received satisfactory answers to all you questions. By signing the consent form you are not waiving your legal rights.

The Ottawa Hospital | L'Hôpital d'Ottawa

Consent Form
Impaired Immune Function during HIV Infection

Consent to Participate in Research

I understand that I am being asked to participate in a research study about immune function in HIV infection. This study has been explained to me.

I have read this, 5-page Participant Information Sheet and Consent Form (or have had this document read to me). All my questions have been answered to my satisfaction. If I decide at a later stage in the study that I would like to withdraw my consent, I may do so at any time.

I voluntarily agree to participate in this study and will provide a:

Please Initial:

_____ Blood Sample

_____ Semen Sample

_____ Gut Sample

A copy of the signed Information Sheet and/or Consent Form will be provided to me.

Signatures

_____
Participant's Name (Please Print)

_____ _____
Participant's Signature Date

Investigator Statement (or Person Explaining the Consent)

I have carefully explained to the research participant the nature of the above research study. To the best of my knowledge, the research participant signing this consent form understands the nature, demands, risks and benefits involved in participating in this study. I acknowledge my responsibility for the care and well being of the above research participant, to respect the rights and wishes of the research participant, and to conduct the study according to applicable Good Clinical Practice guidelines and regulations.

_____
Name of Investigator/Delegate (Please Print)

_____ _____
Signature of Investigator/Delegate Date

Valid until MAR 2 8 2014

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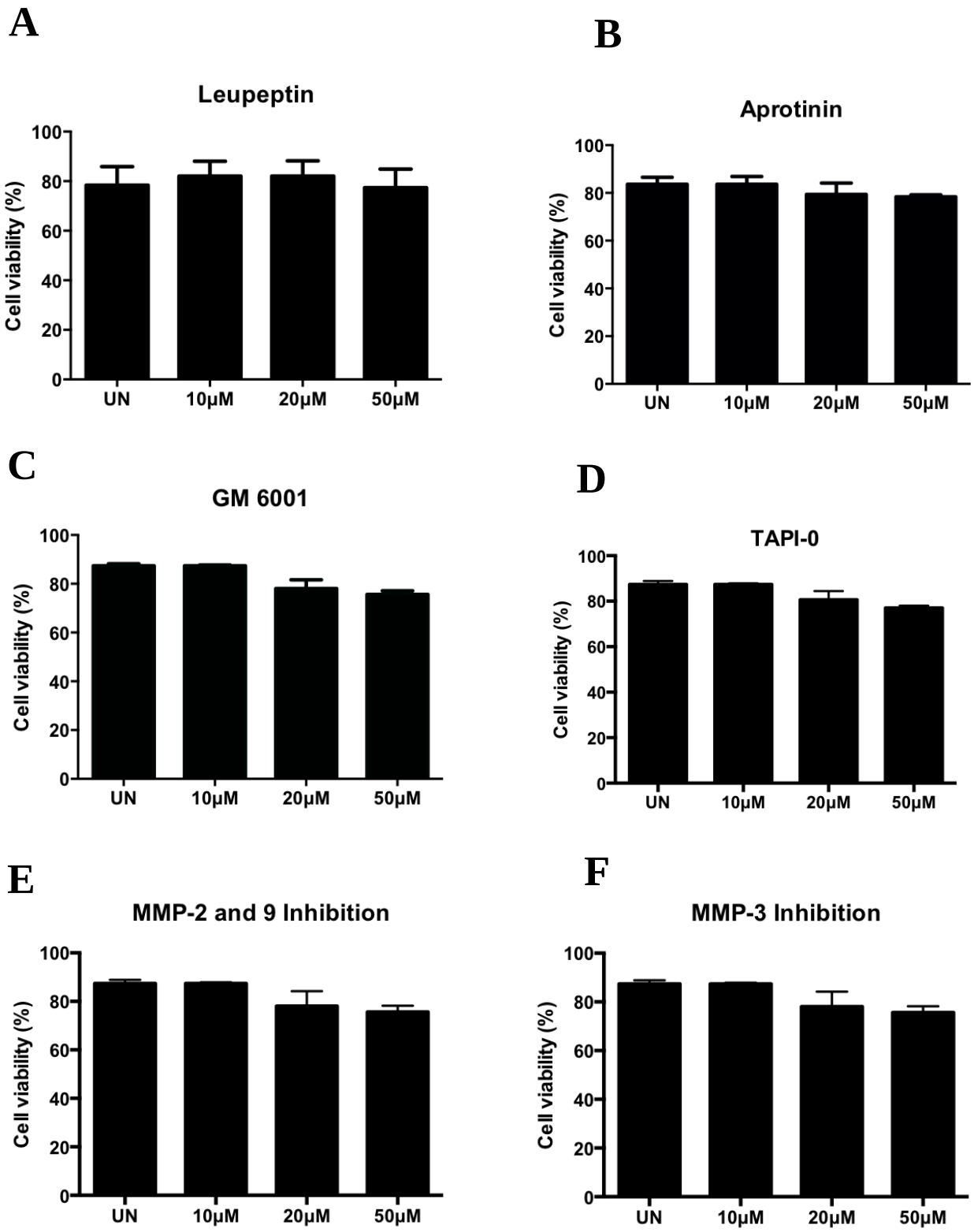
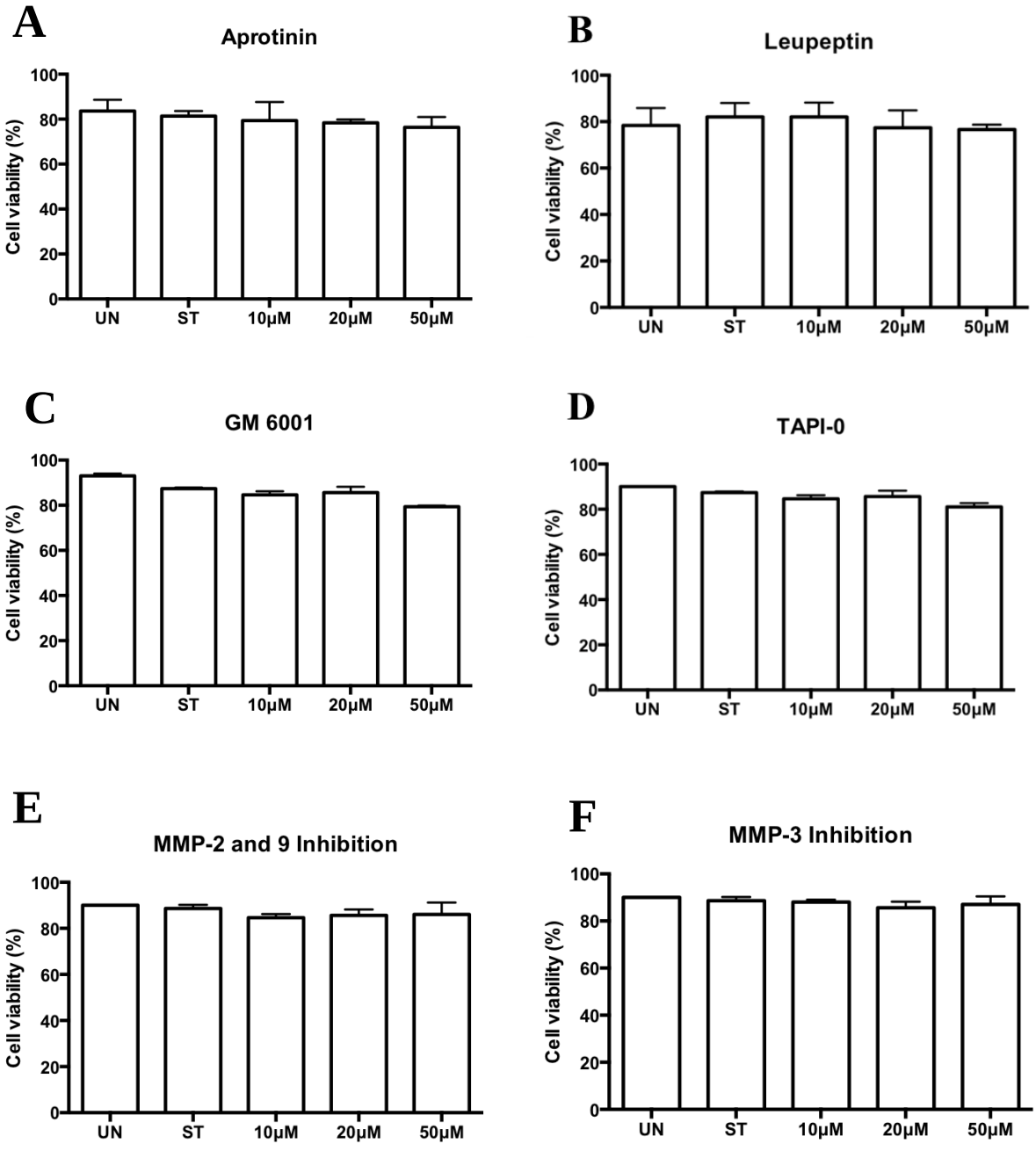
II. Thymocyte viability with different inhibitors

(UN = Media Control; all treated samples were un-stimulated)

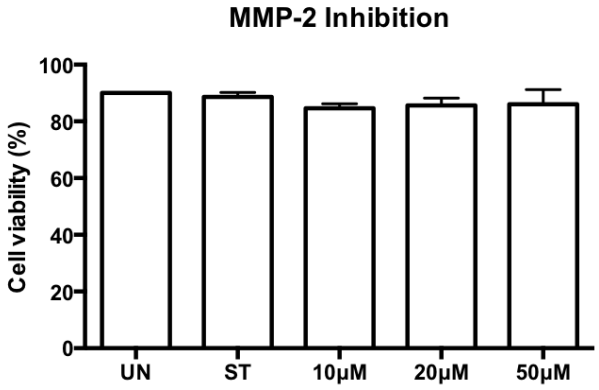


III. $CD8^+$ T-cell viability with different inhibitors

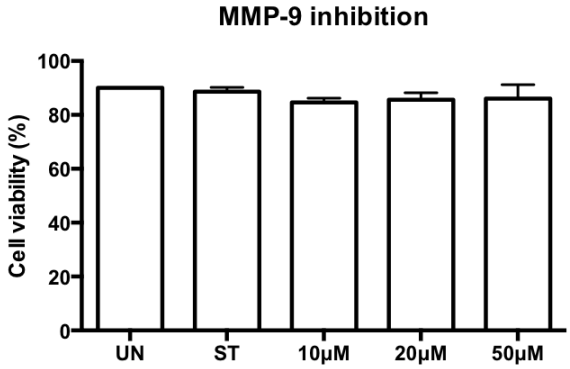
(UN = Media Control; ST = only IL-7/TcR stimulated, no treatment; all treated samples we also co-stimulated)



G


MMP-2 Inhibition
Cell viability (%)
100
80
60
40
20
0
UN
ST
10µM
20µM
50µM

H


MMP-9 inhibition
Cell viability (%)
100
80
60
40
20
0
UN
ST
10µM
20µM
50µM

CURRICULUM VITAE

María Del Mar Sánchez Vidales

Education

University of Ottawa, Ottawa, ON
Master of Science degree in Microbiology and Immunology *05/2013-08/2015*
Major Research Project: Release of Soluble Intrleukin-7 α Receptor (CD127) from $CD8^+$ T-Cells and Human Thymocytes; under the supervision of Dr. Jonathan B. Angel at the Ottawa Hospital Research Institute-Department of Chronic Diseases

Universidad Autónoma de Aguascalientes, Aguascalientes, Mexico
B.Sc. in Biology *09/2006-05/2011*
Major Research Project: Bioremediation of small particles using bivalve mollusks (*Chione undatella*); under the supervision of Dr. Alfonso N. Maeda-Martinez at the Northwestern Center of Biological Research

Work Experience

FIELD TECHNICIAN
Aguascalientes, Mexico *2010-2012*
Animal Health Committee CEFOPAP
Animal and Plant Health Protection Organization from the Ministry of Agriculture (SENASICA-SAGARPA)

Diagnosed illnesses in aquatic organisms and monitored fish farms to assure safe products that are free from pathogens. I routinely visited fish farms and provided recommendations on how to handle their products. In monitoring fish farms, I continuously performed histopathological, microbiological, physical and chemical studies.

SUMMER STUDENT
Riverside, CA USA *2006-2007*
Dr. Frank Wong Laboratory
University of California
Plant Pathology & Microbiology Department

Focus of the project: Discovery and characterization of Xylella fastidiosa strains in southern California causing mulberry leaf scorch and two mutations in β-tubulin 2 gene associated with thiophanate-methyl resistance in Colletotrichum cereale isolates from creeping bent-grass in Mississippi and Alabama.

-Isolated, maintained and stored pathogenic bacteria & fungi, prepared culture media, and preformed DNA extractions and analysis by PCR, ELISA, inoculated plants and maintained greenhouse.

Publications

1. A. Davidson, **M. Sanchez**, Y. Konarski, J.B. Angel, A.M., Crawley. "Mechanisms of soluble IL-7 receptor α (sCD127) generation by CD8+ T-cells. (Submitted for revision: The Journal of Immunology).

2. **M. Sanchez**, Y. Konarski, Carrasco-Medina, L., A.M., Crawley, J.B. Angel. "Soluble IL-7R-alpha is generated by cleavage that involves Matrix Metalloprotease 2 and 9 activity on CD8+ T-cells." (Manuscript in preparation).

Conferences

CAHR (Canadian Association for HIV Research)
- presented poster on original research *2014 & 2015*

OHTN (Ontario HIV Treatment Network)
- presented oral presentation *2014*

OHRI Research Day
- presented poster on original research *2013 & 2014*