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Vitamin C: A potential regulator of inflammatory response

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Vitamin C: A Potential Regulator of Inflammatory Response

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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Dedication

I would like to dedicate this work to my family. Their sincere prayers have always paved (and still pave) the roughness of all my scientific endeavors. My mother, father, brothers, wife, children and all of my family, the near and the far, thank you for your contribution to this work through supporting me emotionally and materialistically.

I would like to dedicate this work to all those who tamed their egos and have been supportive throughout my entire journey. Those who did not hesitate to approach me with supportive criticism as needed. Their love to support the success of others mixed with their experience and scientific wisdom have created the educational platform I always dreamt of. There is no way I can be thankful enough for your time, effort, and on going support. As to those who let the stubbornness of their human nature rule. Those whose egos and opinions were solid/slower to change. You might have layed few obstacles but the benefits far out-weighed the difficulties. I was taught to grow more patient (needed), to speak less (desirable), and let my work speaks (learn more and acquire new skills). Thank you.

Last, as Phi Kappa Phi vice president for graduate student, I would like to share it's motto *"Let the love of learning rule humanity"*.

Acknowledgment

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List of Acronyms and Abbreviations

AA/AscA	Ascorbic Acid
AKI	Acute Kidney Injury
ALI	Acute Lung Injury
ALT	Alannine Transferase
APTT	Activated Partial Thromboplastin Time
Aqp5	Aquaporin 5
Arg 1	Arginase 1
ATF4	Activating transcription factor 4
ATG	Autophagy Related Gene
BAL	Bronchoalveolar Lavage
BiP	Glucose-regulated protein 78
BSA	Bovine Serum Albumin
BUN	Blood Urea Nitrogen
CD	Cluster of Differentiation
cDNA	Complementary DNA
cDNA	Complementary DNA
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
Chi3L3	Chitinase 3 -Like-3
ChiA	Chitinase
CHOP	C/EBP homologus protein

CLnr	Clearance Non Renal
CLr	Clearnace Renal
CRP	C-Reactive Protein
CTGF	Connective Tissue Growth Factor
DHA	Dehydro-ascorbic Acid
DMEM	Dulbecco's Modified Eagle Medium
DTT	Dithiothreitol
EDEM	ER Degradation-enhancing α mannosidase-like protein
EDTA	Ethylenediaminetetraacetic acid
ENaC	Epithelial Sodium Channel
FACS	Fluorescence Assorted Cell Sorting
FBS	Fetal Bovine Serum
FD4	FITC-Dextran
FIP	Feces Induced Peritonitis
Foral	Fraction absorbed
G6PD	Glucose-6-Phosphate Dehydrogenase
Gal-1	Galactin-1
GIT	Gastrointestinal track
GLUT	Glucose Transporters
H&E	Hematoxylin and Eosin
HBSS	Hank's Balanced Salt Solution
HMGB1	Higher Mobility Group Box-1

HnDF	Human neonatal dermal fibroblast
HO-1	Hemoxygenase-1
ICU	Intensive Care Unit
IL-10	Interleukin-10
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-8	Interleukin-8
IND	Investigational New Drug
iNOS	Induced Nitric Oxide Synthase
IP	Intra-Peritoneal
IV	Intra-venous
KC	Neutrophil Chemoattractant Protein
KIM 1	Kidney Injury Molecule 1
LIX	LPS- induced CXC Chemokine
LOX	Lipo-oxygenase
LPS	Lipopolysaccharide
MCP-1	Monocyte Chemotactic Protein-1
MCP-2	Macrophge Inflammatory Protein-2
MMP-9	Matrix Metalloproteinase-9
MO	Macrophages
MODS	Multi-organ Dysfunction Syndrome
MPO	Myeloperoxidase

mRNA	messenger RNA
NET	Neutrophil Extracellular Trap
NETosis	The process of neutrophil extracellular trap formation
NFkB	Nuclear Factor Kappa B
NGAL	Neutrophil Associated Lipocalin
OCT	Octamer-binding Factor
OPDA	O-Phenylenediamine
PAD4	Peptidylargininiedeiminase 4
PBS	Phosphate Buffered Saline
PCT	Procalcitonin
PDGF	Platelet Derived Growth Factor
PMA	Phorbolmyristate acetate
PMN	Polymorphnuclear cells (Neutrophils)
PT	Prothrombin Time
QPCR	Quantitative Polymerase Chain Reaction
RBCs	Red Blood Cells
RDA	Recommended Deitary Allowance
ROS	Reactive oxygen species
RvD1	Resolvin-D1
RvE1	Resolvin-E1
SDS	Sodium Dodecyl Sulfate
SOFA	Sequential Organ Failure Assessment Score

SVCT	Sodium-Dependent Vitamin C Transporters
TCA	Trichloroacetic Acid
TEG	Thromboelastography
TEMPOL	4-Hydroxy-TEMPO or (4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl)
TF	Tissue Factor
TFPI	Tissue Factor Pathway Inhibitor
TG	Thioglycolate
TGF-β	Transforming Growth Factor-β
THP-1	Human Acute Monocytic Leukemia Cell Line
TM	Thrombomodulin
TNF-α	Tumor Necrosis Factor-α
VCAL	VCU Coagulation Advancement Laboratory
VCU	Virginia Commonwealth University
VEGF	Vascular endothelium Growth Factor
VitC	Vitamin C
XBP-1	X-box binding protein-1
XBP-1 un	X-box binding protein-1 unspliced
YM-1	Chitinase 3 -Like-3

Abstract

VITAMIN C: A POTENTIAL REGULATOR OF INFLAMMATORY RESPONSE

By: Bassem M. Mohammed, Ph.D.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2015

Major Director: Donald F. Brophy, PharmD, MS, FCCP, FASN, BCPS, Professor and Chairman, Department of Pharmacotherapy and Outcomes Science

Introduction: Neutrophils (PMNs) and Macrophages are the first responders recruited consecutively to the site of injury/inflammation. PMNs' response/fate as well as macrophage reprogramming ultimately determine the course of resolution of inflammation. Physiologic wound healing has a significant inflammatory component. An exaggerated inflammation however is self-defeating leading to delayed healing. Parenteral vitamin C (VitC) attenuated inflammation in murine sepsis models and in patients with sepsis. However information about the mechanisms by which VitC regulates these events is limited.

Methods: Humanized mice lacking VitC synthesis capability ($Gulo^{-/-}$) were used. VitC *sufficient* and *deficient* mice were challenged with sterile inflammation, or septic insults. Some VitC *deficient* mice received parenteral VitC (200mg/kg) following the challenge to give *deficient + AscA* mice up to 14 days. Using a murine model of excisional wound, two full thickness excisional wounds were created on the back of the different $Gulo^{-/-}$

mice groups. Wound tissues were excised at day 7 and 14 post-wounding for analysis. Cell counts, immunohistochemistry, circulating free DNA, the expression of pro- and anti-inflammatory proteins were investigated. Additional *in vitro* experiments were carried out using human PMN (huPMNs), THP-1 monocyte/macrophage, and neonatal human dermal fibroblasts (HnDF).

Results: VitC *deficiency* delayed resolution of lung inflammation and led to exaggerated pro-inflammatory responses. PMNs from VitC *deficient* mice demonstrated increased autophagy, histone citrullination, and NFκB activation, while inhibiting apoptosis. VitC *sufficiency*/supplementation restored macrophage phenotype, as well as attenuated neutrophil extracellular trap (NET) formation. VitC attenuated pro-inflammatory responses in THP-1 macrophages. In wound healing model, wounds from VitC *sufficient*/AscA infused mice had lower gene expression of the pro-inflammatory mediators; higher expression of genes promoting wound healing and resolution. Exposure of HnDF to AscA increased their intracellular VitC levels; promoted fibroblast proliferation and induced expression of fibroblast self-renewal genes.

Conclusion: Our findings identify VitC as a novel regulator of PMN and macrophage responses. In wound healing, VitC favorably impacted the spatiotemporal expression of transcripts associated with early resolution of inflammation and tissue remodeling. Collectively, these results substantiate the protective notion of parenteral VitC and support its clinical use.

CHAPTER 1. BACKGROUND

1.1 Ascorbic Acid

1.1.1 History and Discovery

Ascorbic acid (AA or AscA) or Vitamin C (VitC) was first isolated in 1928 by the Hungarian scientist *Albert Szent Györgyi* from the adrenal cortex of animals. *Györgyi* was interested in the strong reducing properties of AscA but could not prove its antiscorbutic effects. The isolated molecule was first called hexuronic acid owing to its structure which had 6 carbon atoms and due to its acidic nature. Several years later (1932), a team led by *Charles Gllen King* at the University of Pittsburg was able to isolate AA. *King's* team was trying to isolate the common molecule responsible for treating Scurvy across a number of anti-scorbutics and confirmed that the isolated molecule was in fact *Györgyi* hexuronic acid. The structure of the vitamin was then resolved in 1933 at the University of Brimingham, UK by *Norman Haworth* research team.(1) In 1937, *Györgyi* was awarded the Nobel Prize in *Physiology or Medicine*. In the same year, *Haworth* was also awarded the Nobel Prize in *chemistry* for determining the structure of AA.

1.1.2 Overview

Ascorbic acid (AA) is the reduced and most predominant form of vitamin C (VitC). It is a small organic (Carbohydrate), water soluble molecule, with strong anti-oxidant properties.(2, 3) Its water solubility enables the vitamin to contribute to the body total anti-oxidant capacity both at

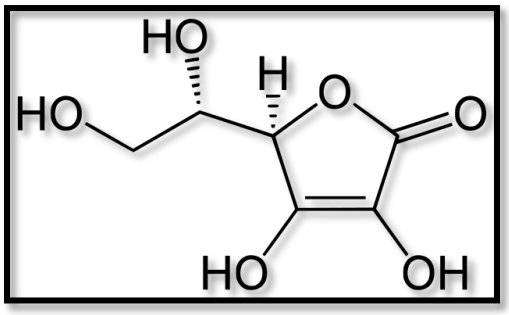

Figure 1: Ascorbic Acid

the plasma level, as well as at the cellular level. Thus protecting host proteins/lipids against oxidative modification via scavenging reactive oxygen species (ROS).

The biological activity of VitC is limited to the more active and naturally occurring (L)-isomer of the vitamin (L-Ascorbic acid). Although, D-isomer has the same anti-oxidant properties, it does not occur in nature. It (the D-isomer) can only be synthesized in a lab setting and does not possess the same biological activity as the L-isomer.(4) When involved in a redox reaction, AA is easily oxidized to the unstable dehydroascorbic acid (DHA).[i] However, DHA is not normally detected in the plasma (but may appear temporarily at times of stress). Data comparing the concentrations of "AA" and "AA + DHA" as total AA, showed no difference.(5)

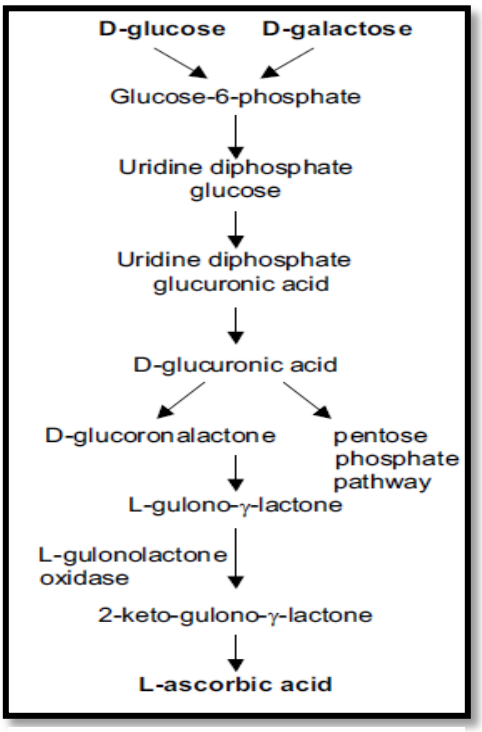

Figure 2: Synthesis Pathway of L-Ascorbic acid

Along with its anti-oxidant properties, VitC is an essential co-factor involved in the enzymatic and non-enzymatic synthesis of several important bodily molecules. This includes: collagen (The most abundant protein in the human body used to make skin, cartilage, tendons, ligaments, and blood vessels), carnitine (an important metabolite needed for fatty acid transfer into the mitochondria for energy production), and neurotransmitters (such as catecholamines).(6)

[i] AA, AscA, and VitC abbreviations will be used interchangeably in the text to refer to L-Ascorbic acid

As to the vitamin sources, the vast majority of animals are able to synthesize their own requirement of VitC using the pathway shown in Figure 2 above. However, humans and few animal species (e.g. guinea pigs, bats, and other primates) have lost this capability. A genetic mutation developed overtime led to the loss of the enzyme *L-gulono-γ-lactone oxidase*. This enzyme executes the final step of VitC biosynthetic pathway. Therefore, Humans and those few animal species are dependent upon external supply in diet to maintain their daily requirement of VitC.(2, 3) This genetic flaw was referred to as *Hypoascorbemia* and was first described by *Dr. Irwin Stone* late in the 20th century.(7)

A severe prolonged VitC deficiency results in a pathological condition known as Scurvy. Scurvy is characterized by alteration of extracellular matrix leading to fatigue, gingivitis, bleeding gums, nose bleeds, ease of bruising, dry/splitted hair, dry/scaly and rough skin, and higher susceptibility to infection. Vision problems, bone fragility, and neurological disorder are also encountered in severe cases of scurvy. Most of the symptoms are due to inactivation of 2-oxoglutarate dependent dioxygenases enzymes as well as other AA dependent enzymes. These enzymes catalyze hydroxylation, desaturation, and oxidative ring-closure or expansion in various major biochemical pathways.(6)

1.1.3 Pharmacokinetics (Absorption/Distribution/Metabolism/Elimination)

As mentioned earlier, AA is the predominant form of VItC. AA is readily absorbed from the intestine and widely distributed into the different body tissues. The body pool of AA is reported to be nearly 20 mg/kg (1400 mg for 70 kg person).(8, 9) Symptoms of

Scurvy are evident with a body pool less than 4.3 mg/kg (< 300 mg for a 70 kg person).(10) Figure 3 shows AA content of plasma and several body organs.(11)

AA is present free in the plasma; data from centrifugal ultrafilteration experiments showed that AA exhibits no protein binding.(5) Its oral absorption is a saturable process; higher doses yield lower F_{oral} (Fraction of drug absorbed). AA administration with food or as a divided dose (versus single dose) improves AA F_{oral} within a dose range of AA.

AA absorption is an active process requiring transporters that are located primarily at the proximal part of the intestine. This was first pointed out with food studies where delaying GI emptying resulted in an increased F_{oral}. Transport of vitamin C into tissues is achieved by two sodium dependent vitamin C transporters (SVCT1 and SVCT2). SVCT1 is the predominant transporter in the intestine. In addition to SVCT transporters which are involved in AA active transport, the glucose transporters GLUT1 and GLUT3 are involved in the transportation of the oxidized form DHA via a facilitated diffusion mechanism. GLUT transporters thus provide entry of DHA to cells lacking SVCT(s) such as RBCs.(12, 13) Older studies have reported SVCTs transporters Vmax to be approximately 70 uM with transporter Km of 5 -30 uM.(14) In a more recent review, Km values for SVCTs were reported to range 65 – 237 uM for SVCT1; and 8 – 113 uM for

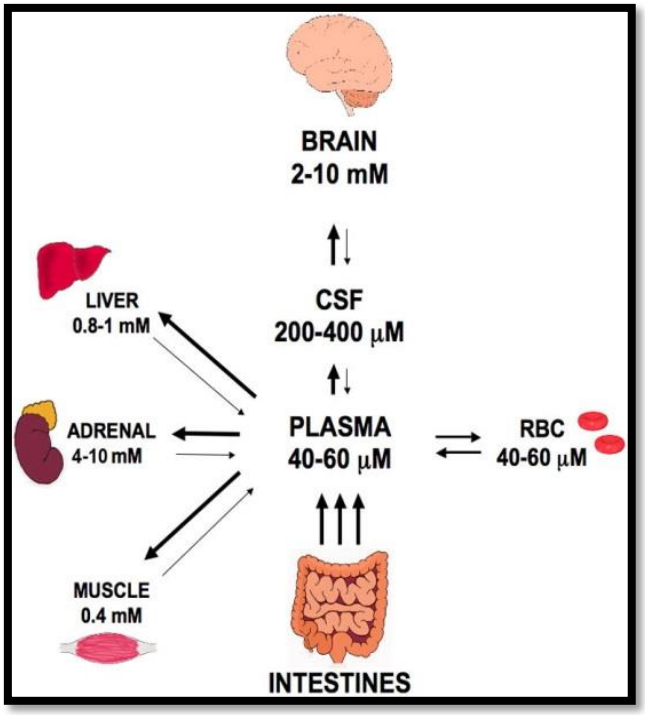

Figure 3: Various organs and plasma AA content. Adapted from reference 11

SVCT2 in human models with SVCT1 having higher Vmax as compared to SVCT2.(13) SVCTs are specific for L-AA transport with very low or no affinity to other forms of the vitamin (eg. D-AA and DHA). SVCT 1 and 2 expressions vary across organs and tissues signifying no redundancy in function.(13)

In addition to saturable absorption, AA renal clearance (CL_r) is shown to follow nonlinear pattern with a saturable re-absorption mechanism. At lower doses (producing plasma AA levels below 70uM), CL_r is low and the reabsorption is not saturated and half-life is reported to be between 8 to 40 days.(15, 16) As the doses increase beyond renal threshold for reabsorption, most of the AA dose is eliminated renally and the non-renal pathways contribution diminishes (CL_{nr} becomes negligible).(17)

As to AA metabolism, the only detectable metabolites of ascorbic acid are dehydroascorbic acid (result of reversible oxidation), 2,3-diketogulonic acid, oxalate (inactive), saccharo-ascorbic acid and ascorbate-2-sulfate (inactive). Carbon dioxide was also reported as a metabolite of AA at doses > 180mg.(18) All of these compounds have been isolated and identified in urine. Ascorbic acid is also excreted in the bile but there is no evidence for enterohepatic circulation.(9) The average fecal excretion of ascorbic acid or its metabolites was found to be approximately 3% of the oral dose when given in physiological amounts. A negligible amount of labeled material (less than 1% during 10 days) was found in the feces after intravenous injection of (l -^{14}C) ascorbic acid. Thus, in humans the principal route for elimination of AA metabolic products is the urine via the kidneys. Daily urinary excretion consists of about 20 - 25% as unchanged ascorbic acid and dehydro-ascorbic acid, about 20% as diketogulonic acid and about 40

- 45% as oxalate. Ascorbic acid 2-sulphate and saccharo-ascorbic acid contribute only very small percentages.(9, 18)

AA Plasma levels: Clinical pharmacokinetics showed that AA levels in plasma and tissues are tightly controlled. At an oral dose of > 100mg/day, the plasma concentration plateau between 70uM and 80uM. Ingestion of larger doses has been associated with minimal change in these values (Figure 4). It is also reported that supplementation near the maximum tolerated dose (2-4gm per oral) yields a plasma level of <250 uM and more frequently <150uM. This ceiling effect is brought about by saturable absorption in the intestine and saturable renal re-absorption as mentioned earlier.(16, 19)

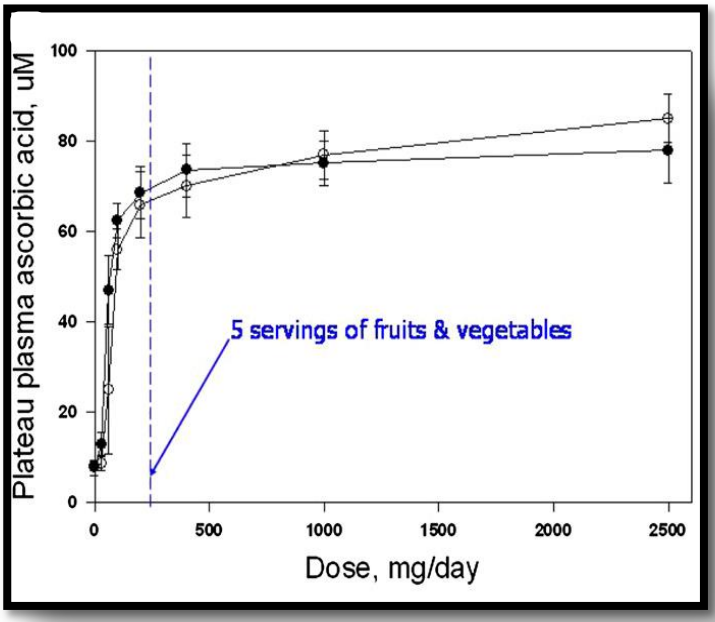

Figure 4: Ascorbic acid dose – plasma concentration curve adopted from reference 19

The recommended daily allowance (RDA) for AA[ii] is calculated based on the average daily intake needed to meet the requirement of all healthy individual. However, RDA is not sufficient when individuals are under the stress of a disease or injury. AA Pharmacokinetics studies showed that when vitamin C is taken orally, plasma and tissue concentrations in healthy individuals are tightly controlled by at least 3

[ii] Acceptable serum levels in mg/dl units are 0.4 to 1.5 mg/dl. In the United States, the recommended adult dietary allowance is 60 mg/day. This amount reportedly maintains the body pool and serum levels of 0.75 mg/dl in normal adults.(8)

mechanisms: absorption, tissue accumulation, and renal reabsorption. A more recently emphasized 4th mechanism is the rate of utilization which comes into play under stress states (e.g. disease, trauma, surgery…etc.)(19) In such cases, increased oral supply or parenteral administration of higher doses are deemed necessary.

Recommended Dietary Allowances (RDAs) for Vitamin C (20)				
Age	**Male**	**Female**	**Pregnancy**	**Lactation**
0–6 months	40 mg*	40 mg*		
7–12 months	50 mg*	50 mg*		
1–3 years	15 mg	15 mg		
4–8 years	25 mg	25 mg		
9–13 years	45 mg	45 mg		
14–18 years	75 mg	65 mg	80 mg	115 mg
19+ years	90 mg	75 mg	85 mg	120 mg
Smokers	Individuals who smoke require 35 mg/day more vitamin C than nonsmokers.			
*Adequate intake				

Last, to provide perspective on AA requirements, animals that can synthesize AA have larger AA body pool. For examples, rats were reported to synthesize ~26 mg/Kg/day; Dogs ~81 mg/Kg/day; Goat ~190 mg/Kg/day. The synthesized amounts are also significantly increased when under states of stress due to a disease or injury. A simple extrapolation to a 70 kg body mass showed that between 1.8 gm/day and 13.3 gm/day will be synthesized/needed. Human RDA on the other hand is around 100mg/day and the maximum plasma AA levels that can be reached with the oral route is below 240 uM which is enough to maintain a body pool of 1.4 gm/70kg.(21) Changing RDA will lead to minimal plasma AA changes, since as described before oral route and the renal elimination threshold limit further plasma AA increment beyond that achieved with RDA. Comparing human and animal data clearly point out what could be a

limitation in the human AA homeostasis preventing optimal therapeutic dosing through the oral route especially under conditions of stress.

1.2 Ascorbic acid and clinical disease states

Under stress, the affected organs/tissues concentrate AA leading to decrease vitamin levels in the circulation. Low/Undetectable AA levels signify a set of dynamic processes involving AA utilization and re-distribution. Re-distribution of AA body pool and high consumption occur as early as the stressful state occur and is sustained as long as it continues. Re-distribution takes place in a way that favors replenishing of the affected tissues, thus increasing their anti-oxidant capacity and preventing the uncoupling of the normal catabolic oxidative processes. Uncoupling of these normal processes is a major contributor of oxidative stress. Therefore, following the major event AA starts to reach a new but lower steady state in the plasma.(8, 22, 23) Dilutional effects associated with massive transfusion protocols used in major injuries also need to be considered.(24) As well, It has been found that the degree of stress and the patient's initial conditions (e.g. Age, dietary intake, comorbidities) play a major role in determining how much of AA is needed.

AA concentration decreases significantly within few hours in critically ill patients during trauma, shock, sepsis, burns, surgery, postoperatively and with postoperative complications.(24, 25) These conditions are known to increase the turnover of ascorbic acid. Reduction in AA level was not corrected by the oral route because of limited absorption. Moreover, the use of low dose parenteral doses (100 – 1000 mg/day) had failed to restore normal vitamin levels for several days in this patient population.(8)

Concentrations achieved orally, in such cases, may approaches that required for enzyme modulation, but are not enough to scavenge superoxide anion which signifies the importance and advantage of the parenteral route.(26) All of these observations in the last century had led investigators to call for increasing AA supplementation for patient undergoing surgery as early as 1946.(8) Not only acute injuries but also chronic disease states such as diabetes and gastritis has also been associated with reduced plasma AA levels.(8)

Timely achievement of AA homeostasis and the added benefits of AA supplementation have been reported in randomized controlled trials involving trauma patients, major burns, critically ill cardiac patients as well as patients with subarachnoid hemorrhage that employed intravenous (IV) vitamin C supplementation. Doses used were between 1gm every 8hours to 66mg/kg/hr (110gm/24hours for a 70kg patient). A significant improvement in patient outcomes was observed in these studies. There was a decrease in patients' ICU length of stay, hospital length of stay, duration on mechanical ventilation, the inflammation marker C-reactive protein (CRP), wound edema, time to wound healing and an overall decrease in morbidity and mortality.(25) In addition, recent research in cardiac surgery has suggested that the use of Vitamin C decreases atrial fibrillation.(25) It is clear that an exaggerated inflammatory response with an oxidative stress self-perpetuating element is central to all of these insults. Indeed, reactive oxygen species (ROS) burst is dominant in critical illness - both acute and chronic - and predispose to multi-organ dysfunction syndrome (MODS), worsening of clinical outcomes, and death.(8)

The sometimes controversial clinical trial results associated with the use of AA could be in part due to the use of different routes of administration. Many of the studies reporting lack of efficacy have employed AA orally in a relatively low dose, which is thought to be less effective. In contrast, when administered intravenously higher plasma concentrations (many folds higher-milliMolar concentrations) are achievable and outcomes are more likely to be affected favorably. Evidence from the literature together with previous work in our labs have shown that there are additional pharmacological benefits that can be reaped when AA levels are in the low millimolar (mM) range (1-3 mM) during periods of stress due to injury or disease.

Lastly, a major fraction of plasma AA is redistributed to immune cells especially neutrophils, macrophage, and lymphocytes. These cells concentrate AA many folds significantly higher than plasma and are major effector cells in the inflammatory response that is known their high utilization of the vitamin. (27, 28)

1.3 Ascorbic acid as a modulator of inflammation (Hands-on experience)

In the span of the last few years, collaborative work between Dr. Natarajan and Fowler, and the Brophy laboratories (VCU advancement Coagulation Lab “VCAL”) have resulted in several in-vitro preclinical studies and a Phase I clinical trial. These series of studies were executed to examine the role of endogenous physiological levels of AA as well as verify the beneficial effect of short term parenteral high dose AA supplementation; explore possible mechanistic pathways regulating these effects; and lastly advocate the clinical usage of this intervention as an add on therapy. Following, in this section, is a discussion of the results of 3 in-vitro studies and the phase I clinical trials conducted in our Laboratories at VCU and the VCU health systems hospital.

Study #1: *"Ascorbic acid attenuates lipopolysaccharide induced acute lung injury"*.(29) In this study, the running hypothesis was that AA attenuate lipopolysaccharide (LPS)-mediated acute lung injury by inhibiting NFkB activation. The research team was able to show that parenteral AA and DHA both at a dose of 200mg/kg were able to prolong survival versus 100% mortality in mice which were only exposed to LPS (Figure 5). AA treatment was shown to preserve the lung architecture as well. Sequestered neutrophil in the lung remained limited to the capillaries with limited to no migration into the alveolar spaces in untreated mice. It also preserved the lung microvascular barrier as evident by less protein content detected in the bronchoalveolar lavage. AA also attenuated the exaggerated neutrophil response and sequestration as evident by the reducedm RNA expression of myeloperoxidase. Looking into the lung NFkB dependent pro-inflammatory cytokines mRNA expression, AA was found to attenuate the augmented increase in KC (Neutrophil chemoattractant factor), LIX (LPS-induced CXC chemokine), MCP-1 (Monocyte chemotactic protein-1), and MCP-2 (Macrophage inflammatory protein-2) expression besides attenuating NFkB signaling itself and diminishing its translocation into the nucleus in the lungs. Hematoxylin and Eosin (H&E) staining of lung sections showed that AA treatment

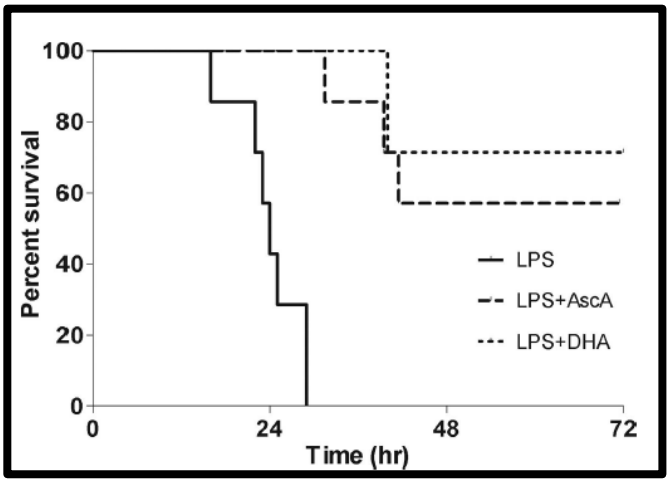

Figure 5: Ascorbic acid (AscA) and dehydroascorbic acid (DHA) prolong survival in septic mice after lipopolysaccharide (LPS) administration. C57BL/6 mice were injected intraperitoneally with LPS (10 µg/g). AscA or DHA (200 mg/kg) was administered intraperitoneally in saline 30 mins after treatment with LPS. LPS vs. LPS + AscA, $p < .001$, log-rank analysis; LPS vs. LPS + DHA, $p < .001$, log-rank analysis (n = 7 for each group)

attenuate sepsis induced coagulopathy and microvascular thrombosis. Areas of microthrombi formation (calculated as % of lung) were significantly reduced following AA or DHA treatment vs untreated lungs in LPS-induced sepsis model. Moreover, AA or DHA treated septic mice had prothrombin time (PT) and activated partial thromboplastin time (APTT) values comparable to the controls. On the contrary, untreated septic mice had significantly prolonged PT and APTT. These results collectively signify blood coagulation factors consumption within the septic mice vasculature underlying an ongoing disseminated intravascular coagulopathy which could be attenuated if the mice were rescued with either AA or DHA i.p. injections following the LPS challenge. On a more molecular level, tissue factor (TF) mRNA expression was also reduced following LPS induced sepsis in the AA or DHA treated groups versus untreated mice. Tissue factor expression is known to be in part regulated by NFkB activation status. Therefore, attenuation of TF expression could either be due to NFkB inhibition or another mechanistic pathway. In conclusion, AA and DHA attenuated LPS-induced lung injury by attenuating the inflammatory response following injury and the associated coagulopathies.

Study #2: “Mechanisms of attenuation of abdominal sepsis induced acute lung injury by ascorbic acid”.(30) This study came as logical step after study #1. Study #1 explored VitC supplementation in a more controlled system i.e. endotoxin induced lung injury. In this study, a live septic insult is employed using fecal stem solution (FIP) prepared from mice fecal pellets and containing live micro-organisms and their respective toxin products. The hypothesis was that VitC will protect lungs against peritonitis induced acute lung injury (ALI). VitC supplementation, at 200mg/kg following

sepsis challenge, is expected to restore alveolar epithelial barrier integrity and prevent sepsis induced coagulopathies. VitC deficient mice experiences 100% mortality within 26 hr from the septic insult. On the contrast, animals treated with VitC rescue dose experienced negligible mortality. Also VitC treatment attenuated the exaggerated expression of pro-inflammatory chemokines KC, LIX, MPO, HMGB1. VitC was shown to maintain barrier function as hypothesized. In more details, VitC treated animals exhibited significantly lower wet/dry lung weight tissue compared to VitC deficient animals at 16 hour post insult. Bronchoalveolar lavage (BAL) from the treated mice showed lower FITC-Dextran (FD4) content compared to untreated mice. From a mechanistic standpoint, VitC was found to prevent sepsis mediated disassembly of the Na+-K+ ATPase pump in vitro. VitC treatment also induced the expression of Aqp 5 (aquaporin 5), CFTR (cystic fibrosis transmembrane conductance regulator) and ENaC (Epithelial sodium channel). Similar observations were made when A549 human alveolar epithelial cells were treated with VitC post endotoxin challenge. Lastly in this work, the authors explored the coagulopathies occuring during sepsis. Thromoboelastography (TEG) was done to assess the blood viscoelastic properties. Prothrombin time (PT) and activated partial thromboplastin time (aPTT) were also carried out. Lung tissue expression of tissue factor, tissue factor pathway inhibitor, thrombomodulin, tissue plasminogen activator, and endothelial protein c receptor were examined. Untreated deficient mice coagulation profile parameters demonstrated a more procoagulant profile and showed marks of consumptive coagulopathies. On the other hand, septic mice treated with VitC showed clot forming parameters that were not different from controls on the TEG (with no indication of consumptive coagulopathies),

and a protein expression profile favoring an anticoagulant environment (i.e favoring less clot formation).

Study #3: “Attenuation Of Sepsis Induced Organ Injury By Vitamin C”.(31) Aside from sepsis-induced lung injury (most frequent following sepsis), a more severe septic insult results in multiple organ dysfunction syndrome. Lungs, liver, and kidneys are the most frequent susceptible organs known to manifest sepsis-induced dysfunction. Acute lung injury (ALI) is considered to be the most frequent complication of sepsis followed by liver dysfunction. Patients with sepsis-induced acute kidney injury (AKI) also have much higher mortality compared to either patients with sepsis or AKI alone. In this study, the same animals grouping as study #2 (discussed earlier) were used. However, the FIP dose used to render animals septic were reduced four folds (180mg/ml to 45mg/ml) to allow VitC deficient mice to live long enough through the experimental period. This also demonstrated increased deficient mice susceptibility to FIP-induced sepsis. Immunohistochemistry, molecular, and biochemical analyses used in the study were able to demonstrate several important aspects. First, the FIP model of animal sepsis was able to produce reproducible sepsis and sepsis-induced organ injury. Second, Multiple organ dysfunction (MODS) was attenuated in VitC sufficient Gulo-/- mice and in VitC deficient $Gulo^{-/-}$ mice infused with a single dose of parenteral AscA compared to VitC deficient $Gulo^{-/-}$ mice. Also deficient $Gulo^{-/-}$ mice developed significant abnormalities in the coagulation system and circulating blood cells which was again attenuated in with either mice VitC sufficiency status or infusion with VitC as AscA. In more details, septic lungs of VitC deficient $Gulo^{-/-}$ mice demonstrated an exaggerated pro-inflammatory response and expressed significantly higher mobility group box-1

(HMGB1), cytokine-induced neutrophil chemoattractant factor (KC), matrix metalloproteinase 9 (MMP9) and myeloperoxidase compared to lungs from VitC sufficient Gulo$^{-/-}$ mice subjected to FIP. Deficient septic mice also displayed pulmonary edema, thickened alveolar walls infiltrated with intramural PMNs, and hemorrhage compared to relatively normal histology with few sequestered PMNs, reduced pulmonary edema and an attenuated pro-inflammatory response seen in sufficient mice. FIP exposed VitC deficient Gulo$^{-/-}$ mice infused with intraperitoneal AscA also had significantly attenuated lung inflammation and reduced lung water compared to untreated deficient mice ($p<0.05$). With respect to sepsis-induced AKI, Kidney sections from VitC deficient Gulo$^{-/-}$ mice subjected to FIP showed mild morphological damage, including tubule vacuolation and occasional loss of brush border. These changes were observed in all FIP exposed groups. In contrast to FIP exposed VitC sufficient Gulo$^{-/-}$ mice or VitC deficient Gulo$^{-/-}$ mice infused with AscA, the kidneys of VitC deficient Gulo-/- mice subjected to FIP were more pro-inflammatory (KC) and displayed increased expression of biomarkers of AKI including kidney injury molecule 1 (KIM1), neutrophil gelatinase associated lipocalin (NGAL), chitinase (ChiA), and chitinase 3-like 3 (Chi3L3) (Figure 3B-H). Blood urea nitrogen (BUN) and creatinine levels were reduced in FIP exposed VitC sufficient Gulo$^{-/-}$ mice and VitC deficient Gulo$^{-/-}$ mice infused with AscA. However a statistically significant difference was achieved only with the BUN levels. As to sepsis-induced liver injury, ALT and AST levels following FIP were elevated in the VitC deficient Gulo$^{-/-}$ mice. These were normalized in both FIP exposed VitC sufficient Gulo$^{-/-}$ mice and VitC deficient Gulo$^{-/-}$ mice infused with AscA In addition MMP9, a critical mediator of tissue injury, was elevated in the FIP exposed VitC deficient Gulo$^{-/-}$

mice. In contrast MMP9 expression was attenuated in FIP exposed VitC sufficient Gulo$^{-/-}$ mice and VitC deficient Gulo$^{-/-}$ mice infused with AscA. Another important piece of this study, was the effect of VitC sufficiency and/or infusion (in previously deficient mice) on the septic mice coagulation profile. The viscoelastic properties, as measured with thromboelastography using TEG machines, of blood from the different mice groups were examined. Blood from septic VitC deficient Gulo$^{-/-}$ mice also displayed profound changes in the rate and kinetics of clot formation (K and Angle). These changes were not evident in FIP exposed VitC sufficient Gulo$^{-/-}$ mice. In addition, AscA infusion restored hemostasis in VitC deficient Gulo$^{-/-}$ septic mice through the normalization of these viscoelastic properties and their hematocrit. The favorable changes conferred by VitC were also associated with a reduction in tissue factor (TF) expression and elevated expression of tissue factor pathway inhibitor (TFPI) and thrombomodulin (TM) in the lung tissues of FIP exposed VitC sufficient Gulo$^{-/-}$ mice and VitC deficient mice infused with AscA.

Study #4: "*Phase I safety trial of intravenous ascorbic acid in patients with severe sepsis".(32)* This study is considered our first trial in patients with sepsis using VitC. The study main purpose was to verify the safety of the intervention. Moreover, important information collected during the study supported the protective notions of using AscA treatment. Important to note that although the study was not powered to assess efficacy, the promising results of the study together with previous in-vitro work fueled a now ongoing multi-center NIH-funded study evaluating the efficacy of using intravenous parenteral AscA as an add-on therapy for the treatment of sepsis-induced ALI. This study was a randomized, double blind clinical trial. Patients were randomized

to one of three groups: Placebo (receiving 5% dextrose), Low AscA group (receiving 50mg/kg/day), and a high AscA group (receiving 200mg/kg/day). Daily treatments were divided into 4 doses and treatment was continued for four days. AscA levels in patients on admission were 17.9 ± 2.4uM (compared to normal levels 50 – 70 uM). Levels were not significantly different across the groups on admission. VitC levels rose significantly in the low and high AscA groups reaching 331 uM, and 3082 uM, respectively compared to the placebo group which continued to decline by day 4 of treatment. Sequential organ failure assessment (SOFA) score across groups were initially the same (p>0.05, NS). Over the 4 days of treatment, both AscA groups showed significant steady decline in SOFA scores compared to the placebo group patients, whose score remained around the baseline values. Additionally, three biomarkers were screened for during the

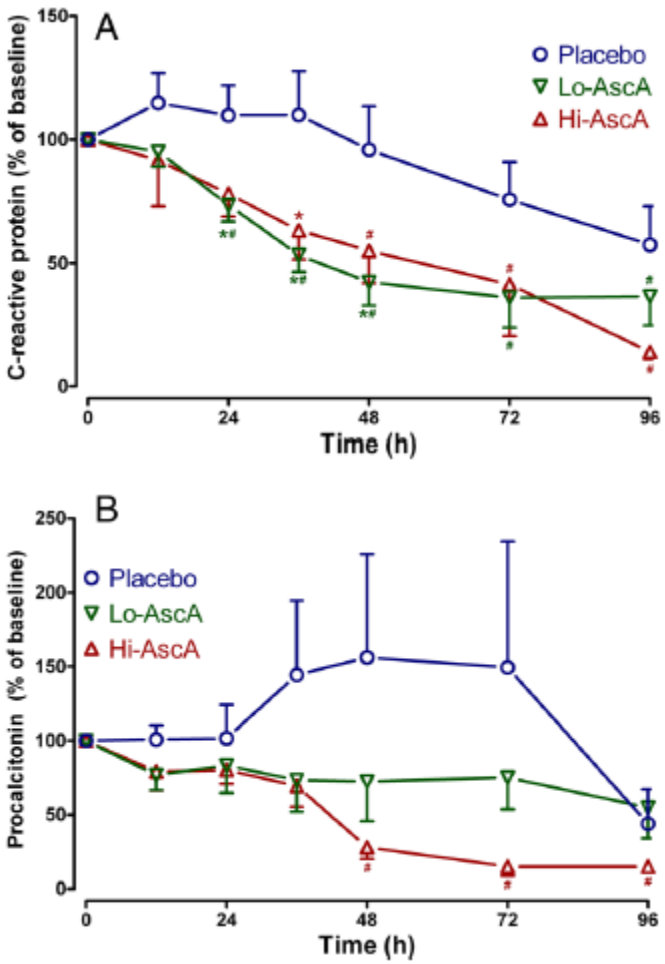

Figure 6: Serum C-reactive protein (CRP) and procalcitonin levels in septic placebo controls and ascorbic acid infused patients. (A) Both the Lo-AscA and the Hi-AscA dosages produced rapid reductions in serum CRP levels, becoming significantly lower than placebo (*p < 0.05 vs placebo) as early as 24 hours. Ascorbic acid infusion reduced CRP levels in both groups throughout the 4 study days (#p< 0.05 vs 0 hr). CRP levels in placebo patients slowly fell over the course of the 4 day study period. (B) Patients in the Lo-AscA and Hi-AscA groups exhibited reduced serum PCT levels beginning at 12 hours. Patients in the Hi-VitC group exhibited further significant reduction in serum PCT between 36 to 48 hours (#p < 0.05 vs 0 hr). Placebo patients exhibited a trend towards increased PCT levels which declined starting at 72 hours post onset of sepsis. Placebo (O), Lo-AscA (▼), Hi-AscA (▲)

study course. C-reactive protein (CRP) and procalcitonin (PCT) were used as surrogate biomarkers (Figure 6) for inflammation, and thrombomodulin (TM) as a surrogate for endothelial injury. Both AscA groups showed a significant rapid drop in CRP at 24 hours and continued during the follow up period. PCT levels were lower in the AscA groups but not significant over the first 24 hours. PCT levels started to decrease in the AscA groups with the high AscA group being significantly lower than placebo past the 24 hr time point and throughout the rest of the follow up period. Low AscA group had lower PCT levels at each timepoint compared to placebo but with no enough evidence to show significance due to sample size and variability constrains. It is also important to note that in the placebo group PCT levels started to increase post the 24 hr point and remained significantly elevated till the 72 hr time point. As to TM, it is initially expressd on endothelial cells where it binds and neutralizes thrombin. Higher soluble TM found in plasma is a marker of endothelial injury. TM levels in patients (Figure 7) randomized to placebo started to rise at approximately 36 hrs and continue to rise throughout the follow up period. In contrast, patients randomized to either doses of ascorbic acid exhibited no subsequent rise in plasma TM. Data spread was considerable and the difference did not reach statistical significance. In conclusion, using the previously described doses of AscA intravenously

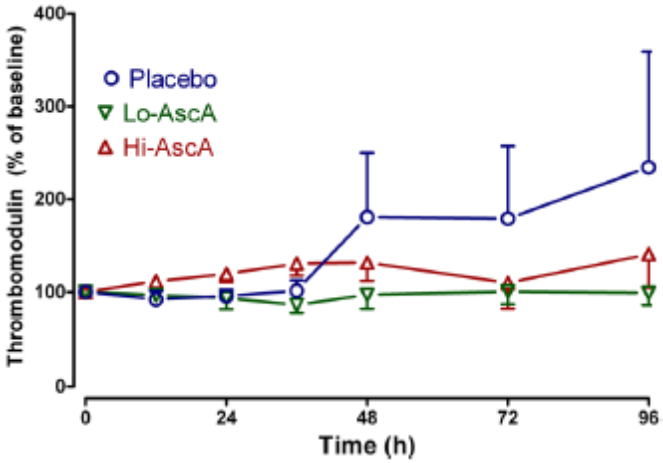

Figure 7: Plasma thrombomodulin (TM) levels measured in septic placebo controls and ascorbic acid infused patients. Plasma TM levels measured in the ascorbic acid infused patients exhibited no rise throughout the 4 days of study. Patients in the placebo group showed a trend towards increased plasma TM levels beginning at 36 hours, though it did not achieve statistical significance. Placebo (O), Lo-AscA (▼), Hi-AscA (▲).

is safe; though the cohort size was small, the data suggested that AscA infusion could significantly attenuate inflammation and endothelial injury inflicted by sepsis.

As mentioned earlier neutrophils, macrophages and lymphocytes and platelets concentrate millimolar quantities of VitC underscoring its essential metabolic role for normal functioning in these types of cells.

1.4 Safety

Short term, high dose, parenteral AA is not only successful at achieving the desired high plasma AA concentration but it is also better tolerated than oral route and associated with less discomfort.(21) In general, AA has a large safety margin and low toxicity profile even at higher intakes.(20, 33)

Several online drug databases as well as the AA material safety data sheet report that the most common side effects associated with AA use are: nausea, diarrhea, gastrointestinal tract (GIT) discomfort and cramps.(21, 33, 34) These side effects are associated with the oral route and are believed to be the result of high osmotic pressure effects that is built up by unabsorbed AA in the GIT.(33, 34) Also side effects like headache and fatigue have been reported. These are acute in nature and usually last less than one day.(21) Diuretic effects have been observed with the use of IV AA and patients should be observed for any signs of dehydration.(35)

AA acidifies the urine. This combined with increased oxalate and uric acid excretion, products of AA and normal metabolism, has been reported to increase the probability of kidney stones formation. Strong evidence to support kidney stones formation with AA intake in the literature is lacking.(21) Intakes from as low as 30 mg and up to 10 gm/day

have been studies with controversial results.(20) May be the two most scientifically sound conclusions in this regard are: 1- AA may contribute to kidney stone formation in patients with an already impaired renal function and/or with pre-esxisting oxalouria; and 2- Higher probability of kidney stones could be associated with continuous intake/infusion over a long period of time (days to weeks) and is limited to oxalate renal calculi.(36, 37)

AA is known to enhance iron absorption. This phenomenon does not adversely affect healthy individuals. On the other hand, individuals with iron overload disorders such as hereditary hemochromatosis, Thalassemia, Sideroblastic anemia, Glucose-6-Phosphate dehydrogenase deficiency (G6PD) and sickle cell anemia need to exercise caution and be screened. An exacerbated iron overload with AA intakes in such cases may lead to tissue damage.(20, 38)

It is important that patients are checked for Glucose-6-phosphate dehydrogenase (G6PD) deficiency before starting treatment with high dose AA. Intravenous high dose of AA can result in hemolysis in such individuals.(39)

Another potential concern was the hypothesized pro-oxidant property of high dose Vitamin C. However, evidence supporting this claim in literature is weak (except at very high doses of.100g/day given IV) and the antioxidant property prevails at the low millimolar range except with increased free iron load.(40)

It is fortunate that the safety of the dose, route, and targeted plasma levels in our studies have been verified in the literature as well as in our preclinical and clinical work.

The general safety profile of even much higher doses has been verified in studies conducted in cancer patients.(41)

To conclude, overall AA is a very safe and low risk intervention over a wide range of doses orally and parentrally. The parenteral route is considered most effective for delivery of high AA levels that bypass the GIT absorption boundaries, thus serves making Vitamin C a “new (emerging) drug” to be used in conjunction with current therapies for patients.

1.5 Setting the stage

This thesis work is formulated into an introduction plus five chapters. All the chapters collectively elaborate on the uses of AA as a modulator of inflammation in varying contexts while providing a mechanistic approach to such findings. Chapters two, three and four are already published work, and are incorporated into the thesis as published. Only difference between the work as presented here and the published version are the figures numbers and citation order. Figures’ number and citation throughout the thesis are flowing as a continuum throughout. Chapter five refers to an in-progress clinical trial [the study has been approved by the institutional review board (IRB), and we are currently in the process of applying for an indication-specific investigational new drug application (IND)]. Each of the chapter/papers/research protocols is divided into subcomponents (introduction, methods, results, and conclusion) and hence complete. Enjoy!

CHAPTER 2: VITAMIN C: A NOVEL REGULATOR OF NEUTROPHIL EXTRACELLULAR TRAP FORMATION

2.1 Introduction

Polymorphonuclear neutrophils (PMN) play key roles in the host response to pathogens by regulating innate host defenses and modulating inflammation. PMN combat pathogens by multiple mechanisms including phagocytosis, followed by exposure to reactive oxygen intermediates (short-lived and long-lived) in phagolysosomes (42), degranulation, which involves release of anti-bacterial peptides and proteases to kill pathogens (43), as well as production of cytokines and other inflammatory mediators. Aside from these traditional mechanisms, another mechanism for pathogen killing, the formation of neutrophil extracellular traps (NETs) by NETosis, a novel cell death pathway different from apoptosis and necrosis, was recently identified.(44, 45) Although NETosis plays a crucial role in host defense during local infection by trapping and killing pathogens, excessive NET formation during systemic infections becomes self-defeating by promoting tissue injury and organ damage.(46)

Sepsis, a leading cause of death with high mortality rates, is characterized by excessive inflammation and exuberant immune responses that lead to increased circulating PMN levels and extensive PMN sequestration in the lung. This massive influx of PMNs to the lungs often leads to acute lung injury (ALI).(47) One postulated mechanism by which PMNs cause ALI is NETosis.(48) In sepsis, NETs are formed in response to pro-inflammatory stimuli such as lipopolysaccharide (LPS) and interleukin-8 (IL-8) (49, 50) by expulsion of genomic DNA into web-like extracellular structures that display

antimicrobial proteins such as histones, neutrophil elastase, and myeloperoxidase.(51) During NETosis, various signaling pathways lead to dissolution of nuclear envelope, thus allowing the mixing of nuclear chromatin with granular antimicrobial proteins from cytoplasmic granules, and then, by releasing the DNA into lattice-like structures, NETs concentrate proteases and antimicrobial proteins in the vicinity of trapped pathogens. However, in sepsis, exposure to NETs also produces organ injury. Indeed, Dwivedi *et al.* recently showed that NETosis, as determined by the circulating cell free DNA (cf-DNA) content, could predict ICU mortality in severe sepsis better than existing severity of illness or organ dysfunction scoring systems and was also better than IL-6, thrombin, and protein C.(52) While effective targeting or inhibition of NET structures has been suggested as therapy to benefit sepsis,(49) identification of agents with the potential to alter NET formation remains elusive.

Vitamin C (VitC) is an essential vitamin for humans. While its role as an endogenous antioxidant is well recognized, our recent research suggests that VitC beneficially impacts multiple pathways associated with sepsis.(29) Its pleiotropic mechanisms including attenuation of the pro-inflammatory response, enhancement of epithelial barrier function, increasing alveolar fluid clearance, and prevention of coagulation abnormalities constitute a primary line of defense that is protective in sepsis syndromes.(30) Intracellular levels of VitC in various tissues differ significantly from circulating plasma levels with high cellular concentrations considered to be indicative of essential metabolic function.(53) In particular, VitC accumulates in millimolar quantities in PMNs where it regulates neutrophil apoptosis.(53, 54) We recently showed that VitC attenuated neutrophiliccapillaritis and improved survival in murine sepsis models.(29,

30) However, whether VitC alters NETosis in sepsis settings remains unknown. Humans lack functional L-gulono-γ-lactone oxidase (Gulo), the final enzyme in the biosynthesis of VitC.(55) In contrast, mice express functional Gulo, resulting in tissues generally maintaining high levels of VitC. In order to translate data from VitC studies in mice to humans we have examined NETosis in septic mice lacking Gulo (Gulo−/−). Our studies show that VitC sufficiency attenuated NETosis in septic mice. Importantly, at a cellular level, we show that VitC deficient PMN were more susceptible to undergo NETosis through increased activation of endoplasmic reticulum (ER) stress and autophagy, processes considered vital for NETosis.(56) VitC deficient PMNs displayed increased expression of peptidylargininedeiminase 4 (PAD4), the key enzyme required for hypercitrullination of histones and chromatin decondensation.(57) Moreover, our studies show that the pro-survival transcription factor nuclear factor kappa B (NFκB) was augmented in the VitC deficient PMNs while apoptosis was suppressed. The inhibitory effect of VitC on NETosis was recapitulated in phorbolmyristate acetate (PMA) activated human PMN.

2.2 Experimental Section

2.2.1 *Animals*

Gulo−/− mice were bred in-house from an established homozygous colony as previously described.(31) Vitamin C *sufficient* mice were fed *ad libitum* with regular chow and water supplemented with vitamin C (0.330 g/L) renewed twice per week. Vitamin C *deficient* mice were generated by reducing vitamin C supplementation (0.033 g/L) for one week, followed by complete removal of dietary vitamin C for an additional

two weeks. Others have shown that this reduced supplementation significantly decreases the concentration of VitC in immune cells, plasma and organs.(58, 59)

2.2.2 Feces Induced Peritonitis

Polymicrobial sepsis (peritonitis) was induced by intraperitoneal (i.p.) introduction of fecal stem solution into the peritoneum as described previously.(30, 31) Thirty minutes after fecal challenge (45 mg/mL), some mice received i.p. injection of VitC as ascorbic acid (200 mg/kg in saline). Untreated mice received i.p. saline instead of VitC. Blood was collected 16 h later by cardiac puncture, and lungs harvested. Blood was allowed to coagulate, spun to separate serum, and stored at −80 °C for batch analysis of cell-free DNA (see below). All animal studies were performed in accordance to the Virginia Commonwealth University Animal Care and Use Committee's approved protocols (Protocol # AM10100, approved 15 March, 2011).

2.2.3 Gulo−/− Mice Were Divided into Five Groups

(1) (**+**): vitamin C *sufficient* mice received saline alone (0.4 mL, i.p.)

(2) **FIP**(**+**): vitamin C *sufficient* mice received fecal stem solution (0.4 mL, i.p.) followed 30 min later by saline (0.1 mL, i.p.)

(3) (**−**): vitamin C *deficient* mice received saline alone (0.4 mL, i.p.)

(4) **FIP**(**−**): vitamin C *deficient* mice received fecal stem solution (0.4 mL, i.p.) followed 30 min later by saline (0.1 mL, i.p.)

(5) **FIP**(**−**) **+ AscA**: vitamin C *deficient* mice received fecal stem solution (0.4 mL, i.p.) followed 30 min later by ascorbic acid (0.1 mL, i.p.)

2.2.4 *Isolation of Mouse Peritoneal Neutrophils*

Induction of an enriched exudate of leukocytes in the peritoneal cavity of mice was performed by i.p. injection of 1 mL of aged, sterile 3% thioglycollate solution.(55) After 16 h, mice were euthanized, and the peritoneal cavity was flushed with 5 mL sterile Hanks' balanced salt solution containing 1% BSA (HBSS). The leukocyte pellet containing ~80% neutrophils and ~20% macrophages was washed with HBSS and resuspended in RPMI-1640 medium. Total cell counts were determined with a hemacytometer. Leukocyte viability was assessed by trypan blue exclusion (>99%). PMNs were then purified by adherence to a plastic dish as described by Tsurubuchi *et al*..(60) Briefly, cells from peritoneal exudate were plated into a 35-mm plastic dish and incubated at 37 °C in 5% CO2 in air for 10 min in HBSS. The cells were washed twice with fresh HBSS to remove non-adherent cells. Although there was loss of some PMNs, which did not adhere to the dish, this procedure eliminated most of the macrophages. Cytochemical staining of adherent cells using HARLECO® Hemacolor® Solution (EMD Millipore, EMD Millipore) revealed that >95% of the adherent cells were PMNs.

2.2.5 *Immunofluorescence and Differential Interference Contrast Imaging of Lung NETs*

Formalin fixed paraffin embedded mouse lung sections (3 µm) were rehydrated and heat induced antigen retrieval performed in 0.01 M citrate buffer pH 6.0 for 20 min. Sections were blocked with 1% normal swine serum (NSS, DAKO, Carpinteria, CA, USA) and incubated with primary antibody #1, rat anti-mouse CD41 (MWReg30, ab33661, Abcam, Cambridge, MA, USA), 1:10 diluted in 1% NSS/PBS overnight at 4 °C. Sections were then incubated with goat anti-rat Alexa Fluor® 488 1:50 (Abcam) in PBS for 4 h followed by incubation with primary antibody #2, rabbit anti-

myeloperoxidase (ab45977, Abcam) 1:10 diluted in PBS overnight at 4 °C. Sections were then incubated with chicken anti-rabbit Alexa Fluor® 647 (Invitrogen, Life Technologies, Grand Island, NY, USA) 1:50 in PBS for 4 h followed by incubation with primary antibody #3, mouse anti-histone H2A (L88A6, Cell Signaling, Danvers, MA, USA) 1:200 in PBS overnight at 4 °C, and then finally incubated with goat anti-mouse IgG1 Alexa Fluor® 594 (Invitrogen) 1:50 in PBS for 4 h. Nuclear counterstain was performed with DAPI (Invitrogen) 1:500 for 5 min and sections mounted with Slow Fade Gold (Invitrogen). Negative controls were run in parallel with nonspecific IgG or specific isotype. Confocal microscopy was performed with a Leica TCS SP2 laser scanning confocal microscopy system of the VCU Department of Anatomy and Neurobiology Microscope Facility. Separate images of optical sections were acquired with filters for Alexa Fluor® (AF) 488, 594, 647 and DAPI. Images were assembled with ImageJ software.

2.2.6 *RNA Isolation and Real-Time Quantitative PCR (QPCR) Analysis*

Isolation of total RNA and real-time QPCR analyses were performed as described previously.(29) Primers used for QPCR are listed in Table 1 below.

Table 1. Murine primers used for Quantitative PCR (QPCR).

Name	**Sequence 5′ to 3′**
ATF4 forward	CCTAGGTCTCTTAGATGACTATCTGGAGG
ATF4 reverse	CCAGGTCATCCATTCGAAACAGAGCATCG
BiP forward	GTGCAGCAGGACATCAAGTTCTTGCC
BiP reverse	TTCCCAAATACGCCTCAGCAGTCTCC
CHOP forward	CACCTATATCTCATCCCCAGGAAACG
CHOP reverse	TTCCTTGCTCTTCCTCCTCTTCCTCC

EDEM forward	GCCCTTTGGTGACATGACAATTGAGG
EDEM reverse	TCATTATTGCTGTCAGGAGGAACACC
XBP-1s forward	TGAGTCCGCAGCAGGTGC
XBP-1s reverse	CAACTTGTCCAGAATGCCCAAAAGG
XBP-1un forward	AAGAACACGCTTGGGAATGGACACGC
XBP-1un reverse	ACCTGCTGCAGAGGTGCACATAGTC
PAD4 forward	ACAGGTGAAAGCAGCCAGC
PAD4 reverse	AGTGATGTAGATCAGGGCTTGG
ATG3 forward	CACCACTGTCCAACATGGC
ATG3 reverse	GTTTACACCGCTTGTAGCATGG
ATG5 forward	ACAAGCAGCTCTGGATGGG
ATG5 reverse	GGAGGATATTCCATGAGTTTCCG
ATG6 forward	CACGAGCTTCAAGATCCTGG
ATG6 reverse	TCCTGAGTTAGCCTCTTCCTCC
ATG7 forward	ACGATGACGACACTGTTCTGG
ATG7 reverse	AGGTTACAGGGATCGTACACACC
ATG8 forward	ACAAAGAGTGGAAGATGTCCG
ATG8 reverse	GGAACTTGGTCTTGTCCAGG
TNFα forward	GATGAGAAGTTCCCAAATGGC
TNFα reverse	TTGGTGGTTTGCTACGACG
IL-1β forward	CTGAACTCAACTGTGAAATGCC
IL-1β reverse	CAGGTCAAAGGTTTGGAAGC
18S forward	GATAGCTCTTTCTCGATTCCG
18S reverse	AGAGTCTCGTTCGTTATCGG

2.2.7 *Western Blot Analysis*

Neutrophil whole-cell and nuclear extracts were isolated for Western blot analysis as described previously.(30) Nuclear extracts were isolated using the NE-PER kit (Pierce Biotechnology, Rockford, IL, USA). Proteins were resolved by SDS polyacrylamide gel

electrophoresis (4%–20%) and electrophoretically transferred to polyvinylidene fluoride membranes (0.2 µm pore size). Immunodetection was performed using chemiluminescent detection with the Renaissance Western Blot Chemiluminescence Reagent Plus (Perkin Elmer Life Sciences Inc., Boston, MA, USA). Blots were stripped using the Restore™ Western Blot Stripping Buffer (Pierce Biotechnology Inc., Rockford, IL, USA) as described by the manufacturer. Purified rabbit polyclonal antibodies to LC3B (L7543, Sigma-Aldrich), cleaved caspase-3 (#9661, Cell Signaling), caspase-3 (#9662, Cell Signaling), p62/SQSTM1 (NBP1-48320, Novus Biologicals), NFκB p65 (sc-109, Santa Cruz Biotechnology), Lamin B (sc-6216, Santa Cruz Biotechnology), and actin (sc-1616, Santa Cruz Biotechnology) were used in this study. Optical densities of antibody-specific bands were determined using Quantity One acquisition and analysis software (Bio-Rad, Hercules, CA, USA).

2.2.8 *Isolation of Human Neutrophils and NETs Release*

Human neutrophils were isolated by density gradient centrifugation and hypotonic lysis.(61) Cells were adjusted to 2×10^6/mL in RPMI-1640, seeded onto 8-well IbiTreat µ-slides (Ibidi #80826), 0.3 mL per well, and allowed to adhere for 15 min. PMNs were VitC loaded by incubating for 1 h with 0.3 mM or 3 mM buffered ascorbic acid (Mylan Institutional LLC, Rockville, IL, USA). Neutrophils were stimulated with 50 nM PMA for three hours at 37 °C. Neutrophil conditioned media were centrifuged at 400× *g* for 5 min and the supernatants used for quantification of cf-DNA.(62)

2.2.9 *Immunofluorescence Staining of Human NETs*

PMNs were fixed with 4% paraformaldehyde, permeabilized with 0.15% Triton X-100 in PBS, and blocked with 5% normal chicken serum (Sigma) in PBS. To stain NETs, slides

were incubated with a mouse monoclonal anti-myeloperoxidase antibody (1:200; Santa Cruz sc-52707) and a secondary Alexa Fluor® 488-conjugated chicken anti-mouse IgG antibody (1:200; Molecular Probes A-21200). After staining of DNA with DAPI, neutrophil-derived NET formation was visualized by immunofluorescence microscopy performed on an Olympus model IX70 inverted microscope outfitted with an IX-FLA fluorescence observation system equipped with a FITC and DAPI filter cubes (Chroma Technology, Brattleboro, VT, USA) through Uplan FI objectives (20×, 60×). Images were captured by an Olympus XM10 digital camera using CellSens imaging software (Olympus America, Melville, NY, USA).

2.2.10 *Quantification of Cell Free DNA*

The levels of cf-DNA in human neutrophil supernatants and mouse serum were quantified using the Invitrogen Quant-iTPicoGreendsDNA assay kit according to the manufacturer's instructions (Life Technologies, Grand Island, NY, USA). Fluorescence intensity was measured on a SpectraMax Gemini XPS microplate reader with excitation at 490 nm and emission at 525 nm, with a 515 nm emission cutoff filter (Molecular Devices, Sunnyvale, CA, USA).

2.2.11 *Statistical Analysis*

Statistical analysis was performed using SAS 9.3 and GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). Data are expressed as mean ± SE. Results were compared using Student-Newman-Keuls test or one-way ANOVA and the *post hoc* Tukey test to identify specific differences between groups. Statistical significance was confirmed at a p value of <0.05.

2.3 Results

2.3.1 *Vitamin C Sufficient Mice Demonstrate Reduced Lung NETs and Lower cf-DNA Following Peritonitis-Induced Sepsis*

We have previously shown that fecal peritonitis promotes PMN infiltration of the lungs in VitC deficient mice.(31) Here we used immunofluorescence staining and DIC microscopy to examine the extent of NETs in lungs of mice following FIP induced sepsis. Immuno-positive staining for platelet CD-41 (green), nuclear histones (red), and myeloperoxidase (grey) are visible in the lungs of saline exposed mice (Figure 8A). No appreciable immuno-positive staining differences were seen in the lungs of saline exposed VitC deficient mice (Figure 8C). FIP induced a mild increase in CD-41 immuno-positivity as well as some cytosolic histone staining (Figure 8B). However, no significant histological changes were evident in the VitC sufficient septic mice. In contrast, FIP induced significant NETs in VitC deficient mice as evidenced by dramatically increased co-staining for platelet CD-41 (green), histones (red), and myeloperoxidase (grey) in the vascular and alveolar spaces of septic mice (arrowheads, Figure 8D). Moreover, extensive extra-nuclear staining of histones (arrows) is also evident in this representative section along with thickened alveolar walls. Importantly, FIP exposed vitamin C deficient mice treated with ascorbic acid exhibited significant attenuation of NETs (Figure 8E).

In order to quantify NETs we determined levels of cf-DNA in the serum of VitC sufficient and deficient mice 16 h after sham treatment or FIP. Levels of serum cf-DNA were significantly elevated in the FIP exposed VitC deficient mice (Figure 8F, 5-fold, $p < 0.05$). Treatment of septic VitC deficient mice with ascorbic acid significantly lowered

the cf-DNA values to control levels ($p < 0.05$). In addition peritoneal neutrophils from vitamin C deficient mice were more susceptible to NETosis than those from vitamin C deficient mice (Supplementary Material section below).

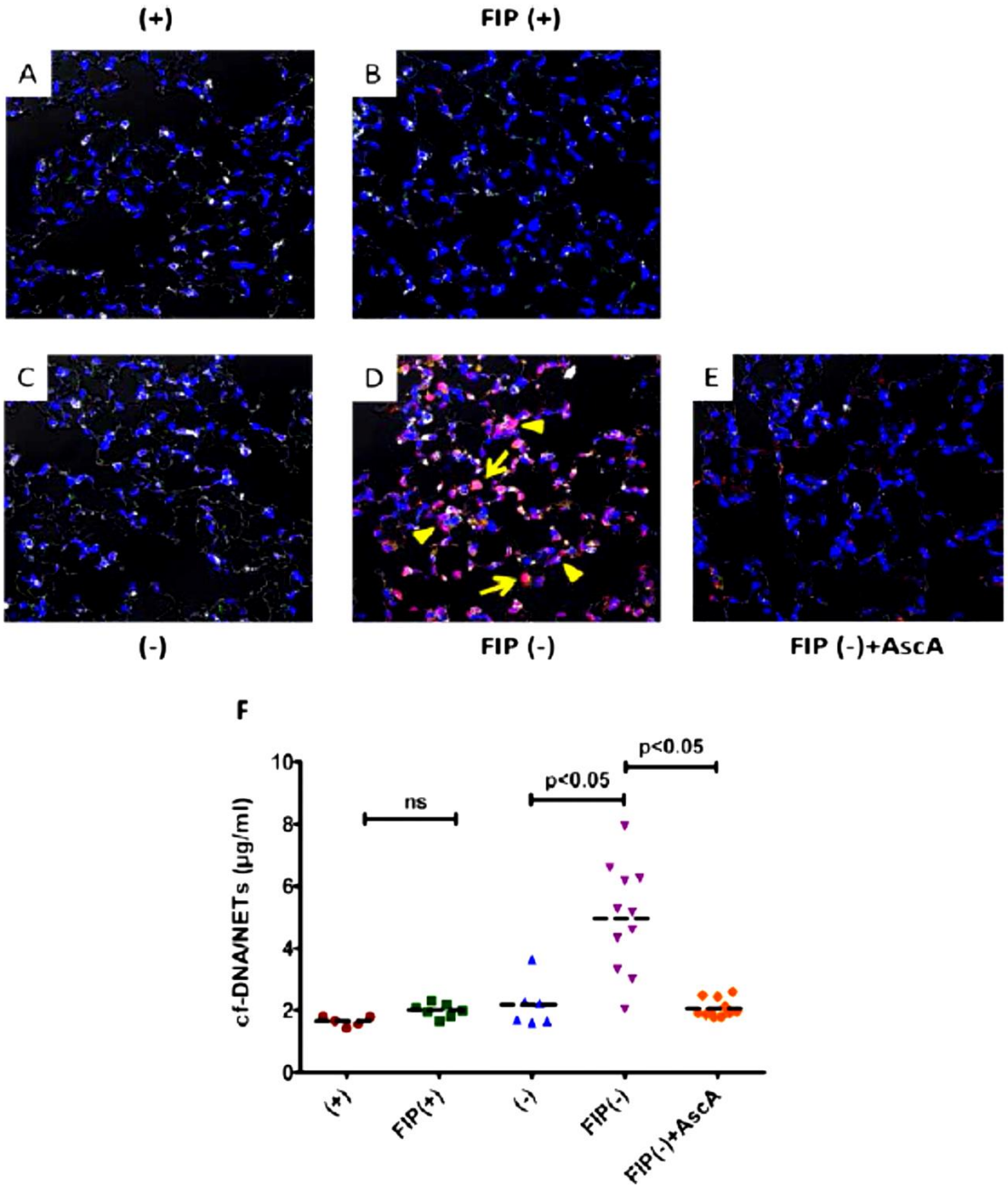

Figure 8 Vitamin C sufficient Gulo−/− mice demonstrate reduced lung NETs and lower cf-DNA following peritonitis-induced sepsis. Representative immunofluorescence and differential interference contrast imaging of lung NETs (A–E): (A) VitC sufficient Gulo−/− mice (+) received saline alone (0.4 mL, i.p.); (B) FIP exposed VitC sufficient Gulo−/− mice [FIP(+)] received fecal stem solution (45 mg/mL, i.p.) followed 30 min later by saline (0.1 mL, i.p.); (C) VitC deficient Gulo−/− mice (−) received saline alone (0.4 mL, i.p.); (D) FIP

exposed VitC deficient Gulo−/− mice [FIP**(−)] mice received fecal stem solution (45 mg/mL, i.p.) followed 30 min later by saline (0.1 mL, i.p.). (**E**) AscA treated FIP exposed VitC deficient Gulo−/− mice [**FIP**(−)** + AscA**] mice received fecal stem solution (45 mg/mL, i.p.) followed 30 min later by AscA (200 mg/kg, i.p.). Platelet CD-41 (green), histones (red), and myeloperoxidase (grey) are seen in the merged images. Arrowheads indicate NET formation shown by co-staining for platelet CD-41 (green), histones (red), myeloperoxidase (grey), and DAPI (blue) in the vascular and alveolar spaces. Arrows indicate extensive extra-nuclear histones (red); (10× magnification, *N* = 3 for each group). (**F**) Serum levels of cf-DNA were quantified using the Quant-iTPicoGreen dsDNA assay kit (*N* = 5–11 for each group, *p* < 0.05).**

2.3.2 *Vitamin C Deficient Neutrophils Show Increased PAD4 mRNA*

Unlike apoptosis, rapid intracellular decondensation of nuclear chromatin is a hallmark of NETosis.(31, 63) Decondensation of nuclear chromatin requires the removal of positively charged arginine residues on histones by deimination or citrullination, which is carried out by a family of peptidylargininedeiminases (PAD). Of these, only PAD4 is expressed by neutrophils (64) and possesses a classical nuclear localization signal.(65) Importantly Wang *et al.* have shown that PAD4 is indispensable for NETosis.(57) Therefore, we examined mRNA expression of PAD4 in PMNs from VitC sufficient and deficient mice. As seen in Figure 9, PAD4 mRNA expression was significantly higher in thioglycollate elicited peritoneal PMNs from VitC deficient mice ($p < 0.05$).

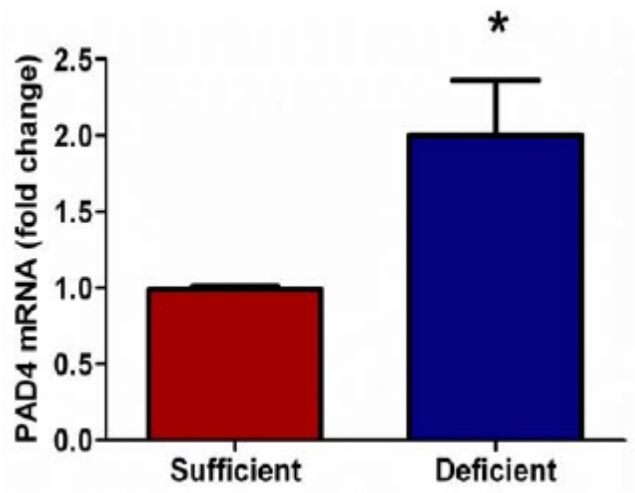

Figure 9 Vitamin C deficient neutrophils show increased PAD4 mRNA. Real time QPCR for PAD4 shows two-fold increase in mRNA expression from peritoneal PMNs of VitC deficient Gulo−/− mice when compared to PMNs from VitC sufficient Gulo−/− mice (*N* = 6 for each group, * *p* < 0.05).

2.3.3 *Autophagy Signaling Is Induced in Vitamin C Deficient Neutrophils*

Autophagy is a vital process for the catabolism of cytosolic proteins and organelles, but has also been shown to be required for NETosis.(56, 66) To examine whether VitC regulates autophagy in PMNs we assessed the expression of several autophagy genes in thioglycollate elicited PMNs from VitC sufficient and deficient mice. As seen in Figure 10A, the expression of autophagy related signaling molecules (except for ATG6) were significantly elevated in the VitC *deficient* PMNs ($p < 0.05$).

Activation of the autophagic process causes lipidation of ATG8/LC3B (LC3B-I to LC3B-II conversion) and the lipid-modified LC3B-II translocates to autophagosomes. This LC3B-I to LC3B-II conversion is considered a critical marker of autophagy activation.(67) We observed significantly enhanced LC3B-I to LC3B-II conversion in cell lysates of VitC *deficient* PMNs by immunoblotting (Figure 3B, $p < 0.05$).

To further investigate the regulation of autophagy signaling by VitC in PMNs, we examined the accumulation of p62/sequestosome I in these cell lysates. The loss of p62 in cells is typically indicative of increased autophagic activity.(68) Detection of p62 by immunoblotting showed a trend towards decreases p62 levels in the VitC *deficient* PMNs (Figure 10C). However this decline did not reach statistical significance ($p = 0.3$).

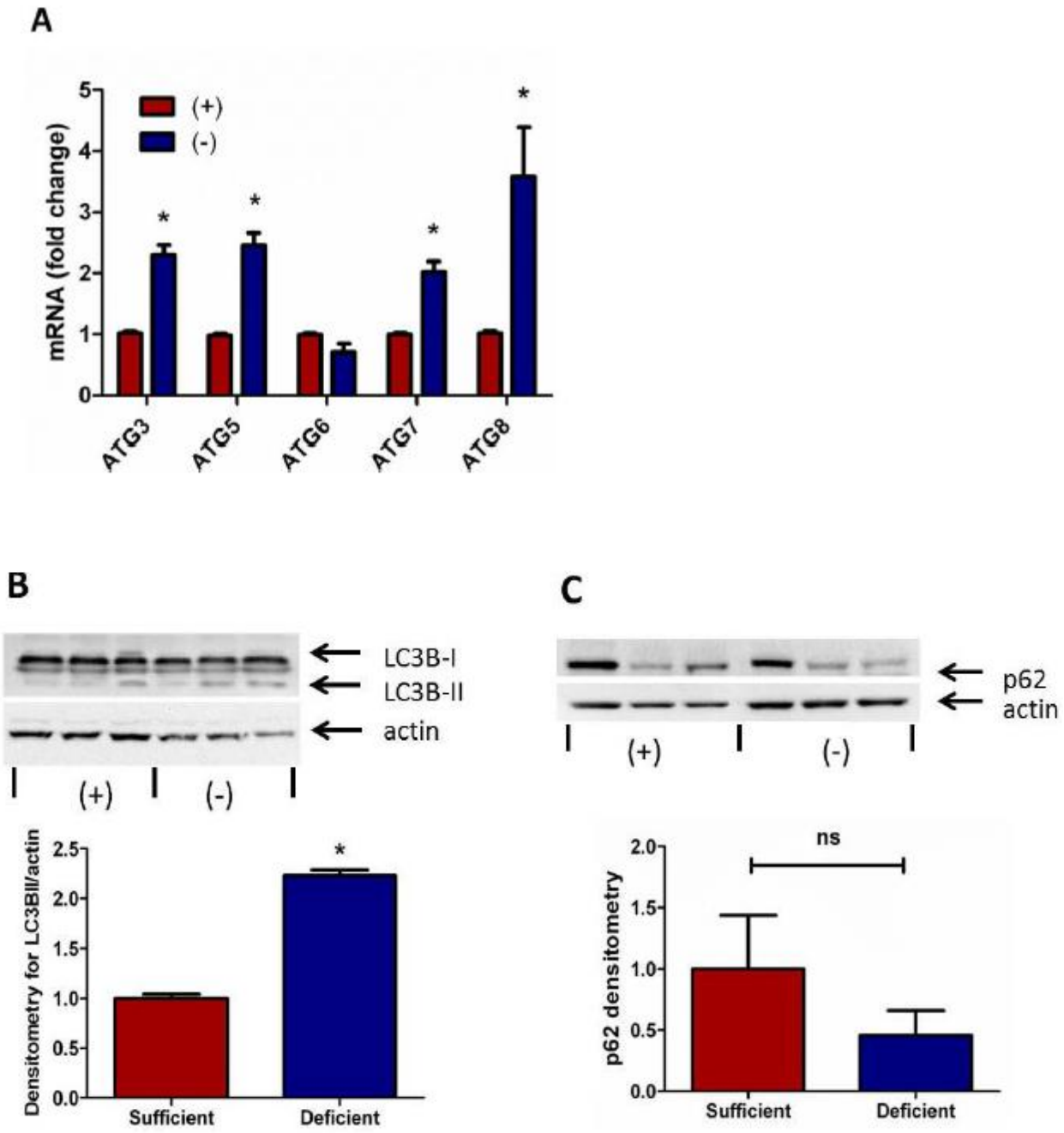

Figure 10 Autophagy signaling is induced in Vitamin C deficient neutrophils. (A**) Real time QPCR for ATG3, ATG5, ATG6, ATG7, and ATG8 mRNA from peritoneal PMNs of VitC sufficient and deficient Gulo−/− mice, (*N* = 6 for each group, * *p* < 0.05). (**B**) Representative Western blot for expression of LC3B-I and LC3B-II from peritoneal PMNs of VitC sufficient and deficient Gulo−/− mice. Densitometry of LC3B-II/actin from peritoneal PMNs of VitC sufficient and deficient Gulo−/− mice (*N* = 6 for each group, * *p* < 0.05). (**C**) Representative Western blot for expression of p62 and actin from peritoneal PMNs of VitC sufficient and deficient Gulo−/− mice. Densitometry of normalized p62 expression from peritoneal PMNs of VitC sufficient and deficient Gulo−/− mice (*N* = 6 for each group, ns *p* = 0.3).**

2.3.4 *Endoplasmic Reticulum Stress Associated Gene Expression Is Up-Regulated in Vitamin C Deficient Neutrophils*

Signaling initiated by the ER stress response (unfolded protein response, UPR) actively participates in autophagy and ultimately contributes to the cell fate decision.(69) Since autophagy signaling was induced in the VitC deficient PMNs, we next examined ER stress gene expression in the PMNs. As seen in Figure 11, all the UPR genes examined except for CHOP were significantly up-regulated in PMNs from VitC *deficient* mice ($p < 0.05$).

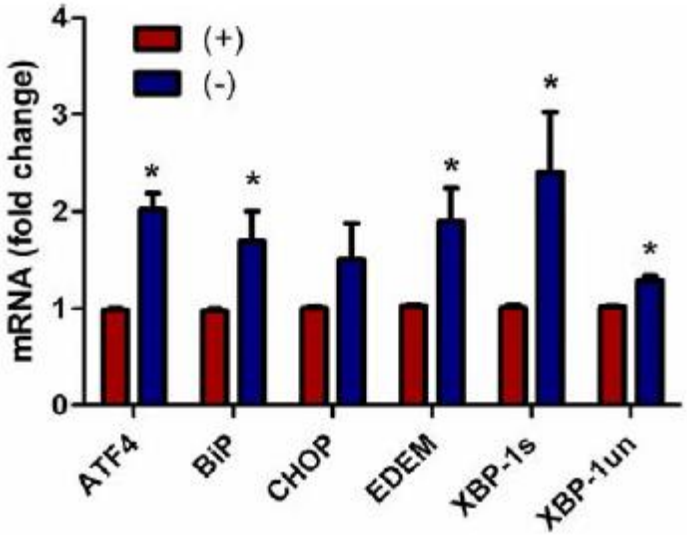

Figure 11 Endoplasmic reticulum stress associated gene expression in up-regulated in vitamin C deficient neutrophils. Real time QPCR for activating transcription factor 4 (ATF4), glucose-regulated protein 78 (Grp78, BiP), C/EBP homologous protein (CHOP), ER degradation-enhancing α-mannosidase-like protein (EDEM), X-box binding protein-1 spliced (XBP-1s), and unspliced (XBP-1un) mRNA from peritoneal PMNs of VitC sufficient and deficient Gulo−/− mice, (*N* = 6 for each group, * *p* < 0.05).

2.3.5 *Vitamin C Deficient Neutrophils Undergo Attenuated Apoptosis*

To determine the extent of apoptosis in peritoneal PMNs from VitC sufficient and VitC deficient mice, we examined a well characterized marker of apoptosis, cleaved caspase-3, by immunoblotting of PMN cell lysates. As seen in Figure 12, caspase-3 activation was significantly lower in VitC *deficient* PMNs ($p < 0.05$).

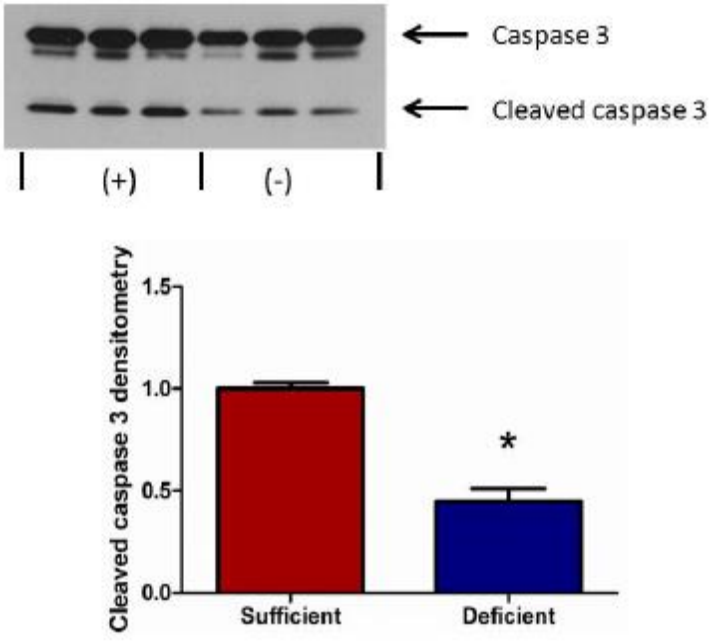

Figure 12 Vitamin C deficient neutrophils undergo attenuated apoptosis. Representative Western blot for expression of caspase-3 and cleaved caspase-3 from peritoneal PMNs of VitC sufficient and deficient Gulo−/− mice. Densitometry of cleaved caspase-3/caspase-3 from peritoneal PMNs of VitC sufficient and deficient Gulo−/− mice (*N* = 6 for each group, * *p* < 0.05).

2.3.6 *Vitamin C Deficient Neutrophils Exhibit Increased NFκB Activation*

The transcription factor NFκB modulates the expression of many immuno-regulatory mediators in the acute inflammatory response in sepsis. Yang *et al.* found that diminished nuclear translocation of NFκB in peripheral PMNs was associated with less time on the ventilator and improved survival in critically ill patients.(70) NFκB activation is also associated with increased ROS production and endoplasmic reticulum stress signaling.(71) Therefore, we examined nuclear translocation of NFκB in peritoneal PMNs isolated from VitC sufficient and deficient mice. As seen in Figure 13A, significantly increased NFκB translocation was observed in nuclei of VitC *deficient* PMNs ($p < 0.05$). Increased nuclear NFκB translocation was associated with induction of the NFκB dependent pro-inflammatory genes for TNFα and IL-1β (Figure 13B, $p < 0.05$).

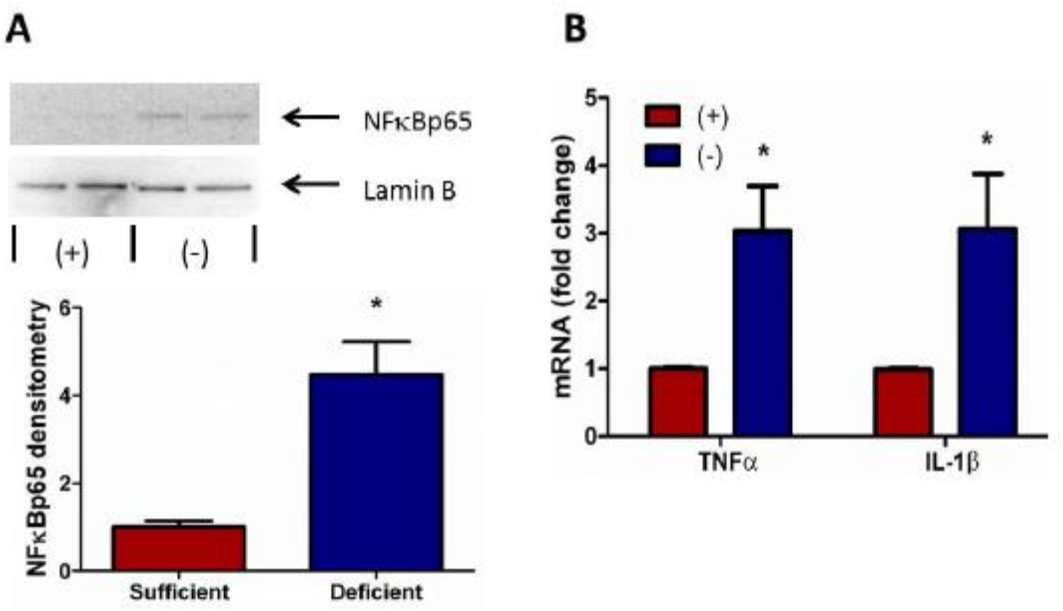

Figure 13 Vitamin C deficient neutrophils exhibit increased NFκB activation. (A) **Representative Western blot for nuclear expression of NFκBp65 and Lamin B from peritoneal PMNs of VitC sufficient and deficient Gulo−/− mice. Densitometry of NFκBp65/Lamin B from peritoneal PMNs of VitC sufficient and deficient Gulo−/− mice (*N* = 4 for each group, * *p* < 0.05).** (B) **Real time QPCR for TNFα and IL-1β mRNA from peritoneal PMNs of VitC sufficient and deficient Gulo−/− mice, (*N* = 6 for each group, * *p* < 0.05).**

2.3.7 *Vitamin C Attenuates NET Formation in Activated Human Neutrophils*

Freshly isolated human PMNs formed NETs following activation by PMA (50 nM) for three hours as seen by immunofluorescence staining (Figure 14B, E). Loading the cells with VitC (3 mM) prior to PMA stimulation greatly reduced NET formation by human PMN (Figure 14C, F). Further, quantification of cf-DNA from the supernatants showed VitC (3 mM) loading significantly reduced NETs release from activated PMN (Figure 14G, $p < 0.05$).

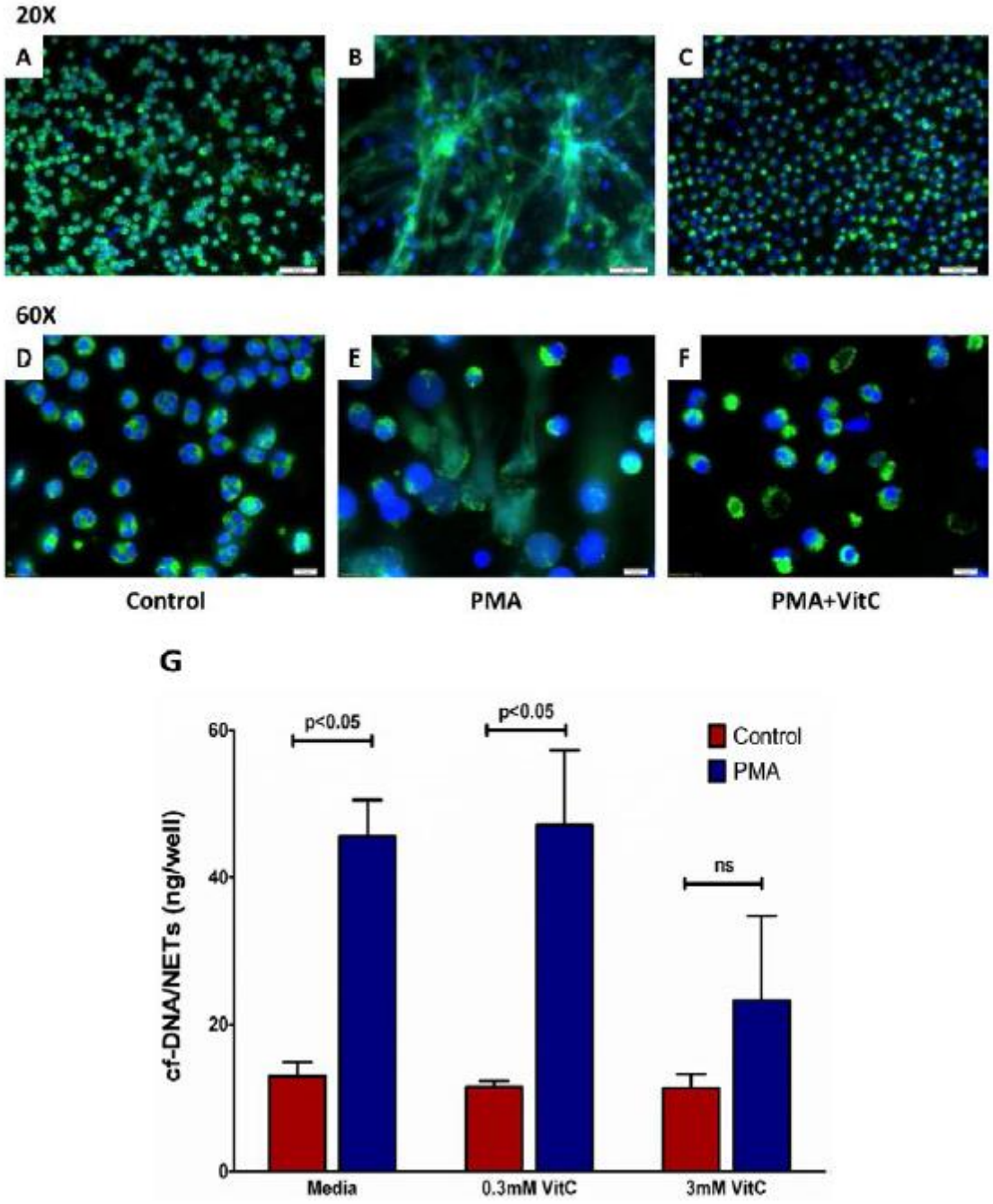

Figure 14 Vitamin C attenuates NET formation in activated human neutrophils. Representative image of immunofluorescent staining for NETs in human neutrophils: DNA (blue); myeloperoxidase (green). Upper Panels**: Control PMNs (**A**, 20×); PMNs exposed to PMA (50 nM) for 3 h (**B**, 20×); PMNs loaded with VitC (3 mM) for 1 h and then exposed to PMA (50 nM) for 3 h (**C**, 20×).** Lower Panels**: Control PMNs (**D**, 60×); PMNs exposed to PMA (50 nM) for 3 h (**E**, 60×); PMNs loaded with VitC (3 mM) for 1 h and then exposed to PMA (50 nM) for 3 h (**F**, 60×). (*N* = 3 for each group, Magnification: upper panel 20×, lower panel 60×). (**G**) Quantification of cf-DNA in the supernatants above (*N* = 3 for each group, *p* < 0.05).**

2.4 Discussion

In this study we show that VitC could play a critical role in regulating the ultimate fate of PMNs in sepsis. Activated PMNs undergo extensive NETosis in septic mice lungs, resulting in potential damage to lung alveolar and endothelial cells. This effect was predominant in PMNs from VitC deficient mice and could be rescued by VitC infusion after the onset of sepsis. In contradistinction, PMNs from VitC sufficient mice underwent attenuated NETosis. Importantly, at a molecular level, VitC deficient peritoneal PMNs were likely to be more pro-inflammatory, to resist apoptosis, and to preferentially undergo NETosis.

Although several signaling mechanisms responsible for NET formation have been reported, critical regulatory elements remain unidentified. This study advances our understanding of PMN function and NET biology by identifying a novel regulatory mechanism for NET formation in both murine and human PMNs. Using our previously well-characterized model of abdominal peritonitis induced sepsis we show that sepsis promotes NET formation in lungs of VitC deficient mice (Figure 8). NETosis in this model was accompanied by increased circulating cf-DNA (Figure 8F). VitC sufficiency or infusion of VitC after initiation of sepsis significantly decreased NETosis in lungs and circulating cf-DNA content (Figure 8). NET formation in VitC deficient peritoneal PMNs required activation of well characterized signaling pathways including ROS generation (data not shown), activation of the peptidylargininedeiminase PAD4 (Figure 9), autophagy (Figure 10), endoplasmic reticulum stress (Figure 11), and inhibition of apoptosis (Figure 12). NFκB, a pro-inflammatory, pro-survival transcription factor was

activated in the VitC deficient peritoneal PMNs (Figure 13). VitC sufficiency or treatment with VitC attenuated these signaling pathways in PMNs.

Intracellular chromatin decondensation is essential for NET formation. Chromatin decondensation is brought about by peptidylargininedeiminase 4 (PAD4), a nuclear enzyme that deiminates arginine residues on histone tails thereby converting positively charged arginines to uncharged citrullines.(56, 57) The importance of PAD4 is that many NET forming stimuli including PMA, LPS, and IL-8 as well as various bacterial and fungal species converge to its activation. While PAD4 is expressed in PMNs and is localized to the nucleus (64, 65), little is known about its mechanism of action or its transcriptional regulation. Ying *et al.* have shown that PAD1, which belongs to the same family of enzymes as PAD4, is transcriptionally regulated by NFκB.(72) We have previously shown that VitC blocks NFκB activation in septic mouse lungs.(29) Cárcamo *et al*. also demonstrated that VitC blocks IκB kinase activity and NFκB activation.(73) In this study we observed that nuclear NFκB levels were higher in the VitC deficient PMNs (Figure 13A). Further, PAD4 mRNA expression was also significantly higher in PMNs from VitC deficient mice (Figure 9). Therefore, we hypothesize that VitC decreases PAD4 expression by suppressing NFκB activation in PMNs. Further, by decreasing PAD4 expression VitC could decrease histone citrullination activity and therefore chromatin decondensation in VitC sufficient PMNs.

Autophagy has been identified as a well-conserved, homeostatic mechanism that clears damaged organelles or proteins and plays an essential role in cell survival during periods of nutrient depletion.(74) Despite the view that it might not occur in neutrophils, autophagy was recently shown to occur both in murine and human PMNs.(75, 76) While

Mitroulis *et al*. reported that autophagy occurs in human PMNs in response to PMA activation.(77) Remijsen *et al*. have shown that autophagy is necessary for the induction of intracellular chromatin decondensation during PMA-induced NETosis.(56) In our study, we found increased expression of autophagy genes (Figure 3A) as well as significantly enhanced LC3B-I to LC3B-II conversion in VitC deficient PMNs (Figure 3B) indicative of the presence of more autophagosomes in VitC deficient PMNs. However, LC3B-I to LC3B-II conversion is a static measure of autophagosome number, and does not measure the actual activity of the pathway. The increased LC3B-II could be interpreted as either high autophagic activity or a downstream block in the system that results in an accumulation of LC3B-II protein, even though autophagic degradation itself is diminished. To supplement our observations we examined levels of p62/sequestosome I, a cytosolic chaperone protein with an LC3B binding domain.(78) The normal function of p62 protein is to carry polyubiquitinated proteins to the autphagolysosome where it binds to LC3B before getting degraded. Thus, the loss of p62 protein is a measure of the flux of autophagy and indicative of increased autophagy.(78) In our studies we found a trend towards decreased p62 levels in the VitC deficient PMNs (Figure 10C). While this decline did not reach statistical significance, in combination with the increased autophagy gene expression and increased LC3B conversion, our data imply increased autophagy in VitC deficient PMNs. The unfolded protein response (UPR) and autophagic machinery have been shown to be critically linked to each other. It is well established that activation of the UPR genes transcriptionally up-regulates several autophagy related genes required for induction and construction of the autophagy machinery.(79) However, it is not known

whether activation of the UPR drives autophagy and eventually leads to NET formation in PMNs. Our study shows that most of the UPR genes examined except for CHOP were significantly up-regulated in PMNs from VitC deficient mice (Figure 11). This implies that VitC deficient PMNs could be actively undergoing ER stress, which in turn could drive autophagy genes and increase their susceptibility to undergo NETosis.

The transcription factor NFκB is central to pro-inflammatory/pro-survival responses in sepsis. It is normally sequestered to IκB in the cytosol. Upon appropriate stimulation, IκB is degraded allowing NFκB to migrate to the nucleus and drives transcription of numerous genes that regulate the immune response in sepsis. Moine *et al*. have demonstrated increased NFκB translocation in the lungs of patients with ALI.(80) Yang *et al*. found that increased nuclear levels of NFκB in unstimulated neutrophils were associated with a worse clinical outcome.(70) As discussed above, NFκB likely drives expression of PAD4 in PMNs. NFκB activation also drives expression of pro-survival genes.(81) In this study we found that nuclear NFκB translocation was higher in VitC deficient PMNs (Figure 13A). Further, NFκB translocation in these VitC deficient PMNs increased expression of the pro-inflammatory genes TNFα and IL-1β (Figure 13). NFκB activation also inhibited apoptosis as seen by the reduced activation of caspase 3 in VitC deficient PMNs (Figure 12). These results suggest that NFκB may play a critical role in modulating cell signaling pathways that eventually regulate the fate of PMNs. By activating PAD4 (chromatin decondensation), inducing ER stress and subsequent autophagy, and inhibiting apoptosis, NFκB may drive the cellular machinery of VitC deficient PMNs towards NET formation (Figure 15). VitC sufficiency or infusion of VitC allows PMNs to increase intracellular levels of VitC and attenuate NFκB activation. This

could dampen the pathways required for NETosis and may allow PMNs to undergo apoptosis instead. While the decreased apoptosis rate in VitC deficient PMNs may benefit the host by giving more time for PMNs to perform their innate immune functions, studies show that it could also be detrimental in sepsis due to the PMN-dependent inflammation and tissue damage that could be heightened by a prolonged lifespan. Recent reports in the literature have implicated NETs in transfusion-related acute lung injury (TRALI), the leading cause of death after transfusion therapy.(82, 83) NETs were shown to be present during TRALI both in mice and humans and so it was suggested that targeting NET formation may be a new approach for the treatment of acute lung injury. While we did not examine TRALI in our studies, it is conceivable that VitC infusion could be a useful adjunct for the prevention/treatment of TRALI or other disease states involving exuberant formation of NETs particularly in the lungs.

Our study has several limitations: (1) It is possible that the PMNs isolated within the peritoneal cavity by thioglycollate could be partially activated; (2) We examined PMN function *ex vivo*. Further *in vivo* studies are needed to characterize the fate of PMNs; (3) others have performed studies with PMNs isolated from bone marrow instead of thioglycollate elicitation. These PMNs would be less “activated” when compared to thioglycollate elicited PMNs, but would also have a large component of immature PMNs which have been shown to behave somewhat differently from mature PMNs.(84)

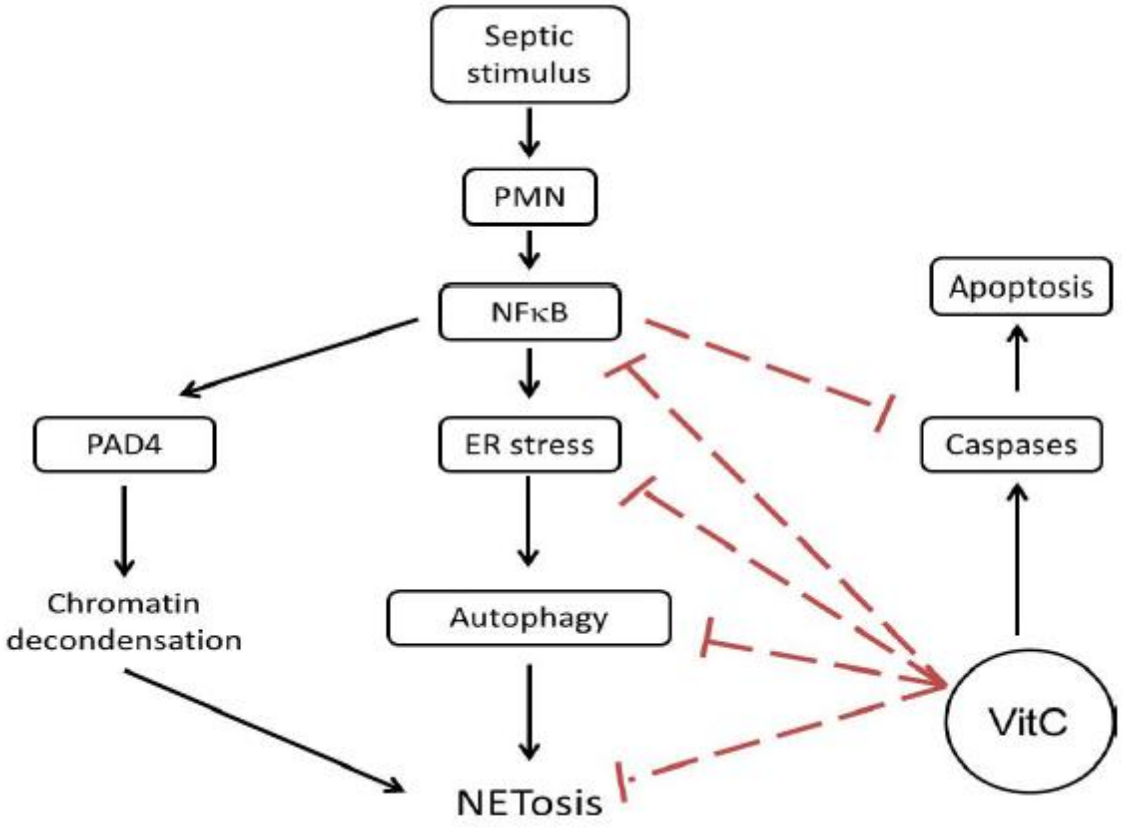

Figure 15 Schematic hypothesis of regulation of signaling pathways that leads to NETosis by VitC. Septic stimuli activate NFκB in PMNs with increased activation observed in VitC deficient PMNs. NFκB nuclear translocation drives expression of PAD4, ER stress and autophagy signaling genes while inhibiting caspase 3 in activated PMNs. This drives the fate of activated PMN away from apoptosis and enhances NETosis. VitC likely blocks up-regulation of PAD4, ER stress and autophagy signaling genes by decreasing NFκB activation. Activated PMNs now undergo apoptosis while NETosis is attenuated.

2.5 Conclusion

In the past few years circulating cf-DNA has been identified as a prognostic marker in severe sepsis.(52, 85, 86) Indeed cf-DNA was shown to have better discriminatory power than IL-6, thrombin or protein C to predict ICU mortality in sepsis.(52) The cellular origin of cf-DNA from host cells was shown by Dwivedi *et al. (52)* who confirmed that the release of cf-DNA was independent of the infecting organism and was likely mediated by inflammatory mediators generated during the exacerbated host immune response. Our study showed attenuated NET formation and reduced cf-DNA in the serum of septic VitC sufficient mice and in VitC deficient mice treated with ascorbic acid

(Figure 1). Our study did not examine the origin of cf-DNA in the serum of these mice. It is possible that some of this DNA could be non-neutrophilic in origin since mast cells, eosinophils, and basophils have also been shown to expel their DNA in a manner similar to PMNs.(87) However, a detailed examination of the origin of cf-DNA in these septic mice is beyond the scope of this study. Nevertheless, data from our study implies that attenuation of NETs maybe crucial for resolution of sepsis in mice.

Overall, our *in vitro* and *in vivo* findings identify a novel regulatory mechanism that limits NET formation in sepsis. These findings implicate VitC as a previously unrecognized layer of regulation that prevents generation of excessive NETs.

2.6 Supplementary Material

Vitamin C (VitC) deficient neutrophils are susceptible to NETosis. In order to determine whether peritoneal neutrophils from VitC sufficient or VitC deficient mice produced NETs without further stimulation, thioglycollate-elicited peritoneal PMNs were seeded onto 8-well IbiTreat µ-slides (Ibidi #80826) and allowed to adhere for 16 h. As described in the Methods section of the manuscript, PMNs were fixed with 4% paraformaldehyde, permeabilized with 0.15% Triton X-100 in PBS, and blocked with 5% normal chicken serum (Sigma) in PBS. To stain NETs, slides were incubated with a mouse monoclonal anti-myeloperoxidase (MPO) antibody and a secondary Alexa Fluor® 488-conjugated chicken anti-mouse IgG antibody. After staining of DNA with DAPI, PMNs were visualized by immunofluorescence microscopy. As seen below (Figure 16a), PMNs from VitC sufficient mice retained their lobulated structure with no significant co-localization of DNA and MPO signals (no inter-mixing of nuclear and cytosolic contents). In contrast, PMNs from VitC deficient mice demonstrated some degree of co-localization of DNA

and MPO signals (Figure 16b). Importantly, nuclei from some of these PMNs (arrows) were delobulated and had released their DNA content into the cytosol. However, full NET structures are not evident in these images. This suggests that thioglycollate stimulation of PMNs is insufficient to induce NETs in murine PMNs from VitC sufficient mice. Conversely, some PMNs from VitC deficient mice were sufficiently activated and had commenced the process of NET formation by intermixing nuclear and cytosolic contents.

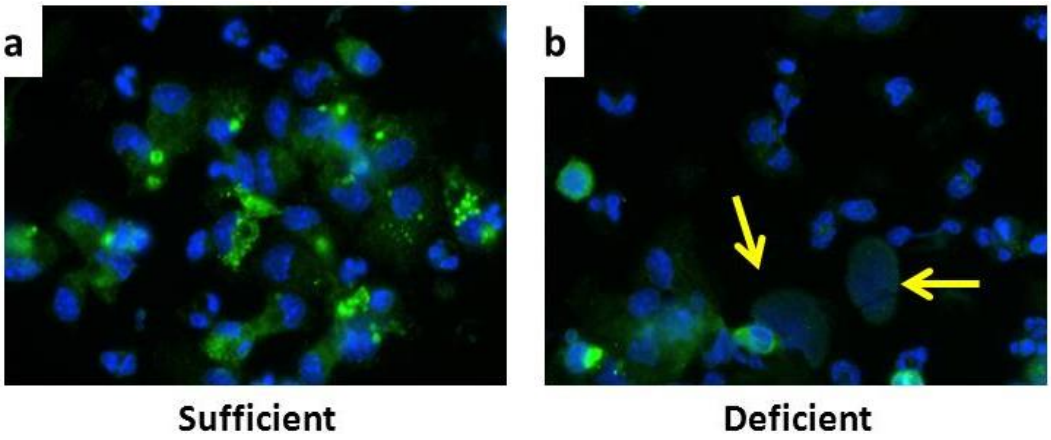

Figure 16 Vitamin C deficient neutrophils are susceptible to NETosis. Representative image of immunofluorescent staining of Thioglycollate elicited peritoneal PMNs (DNA (blue); myeloperoxidase (green)) obtained from: (a) VitC sufficient mice; nuclei from VitC sufficient mice retained their lobulated structure, and (b) VitC deficient mice; PMNs from VitC deficient mice demonstrated some degree of co-localization of DNA and MPO signals. Nuclei from some of these PMNs (arrows) were delobulated and had released their DNA content into the cytosol.

Detection of reactive oxygen species in peritoneal neutrophils by flow cytometry. The generation of ROS and superoxide radicals in peritoneal PMNs was measured by using the cell permeable dye 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA, Invitrogen) as described by Chatterjee et al.(88) Briefly, peritoneal PMNs from VitC sufficient and deficient Gulo$^{-/-}$ mice were re-suspended in cold PBS/ 1% BSA (4˚C) and incubated in the dark for 15 min with 10μM carboxy-H_2DCFDA. Samples were acquired on a BD Accuri® C6 Flow Cytometer (BD Accuri Cytometers,

MI, USA) and mean fluorescence intensity recorded for at least 10,000 neutrophils. Data was analyzed using FlowJo software (Tree Star, Ashland, OR). Results were compared using Student-Newman-Keuls test and the post hoc Mann-Whitney U test to identify specific differences between the groups. There was no significant difference (Figure 17) in the amount of ROS produced by peritoneal PMNs isolated from VitC deficient/sufficient Gulo$^{-/-}$ mice (N=8/group).

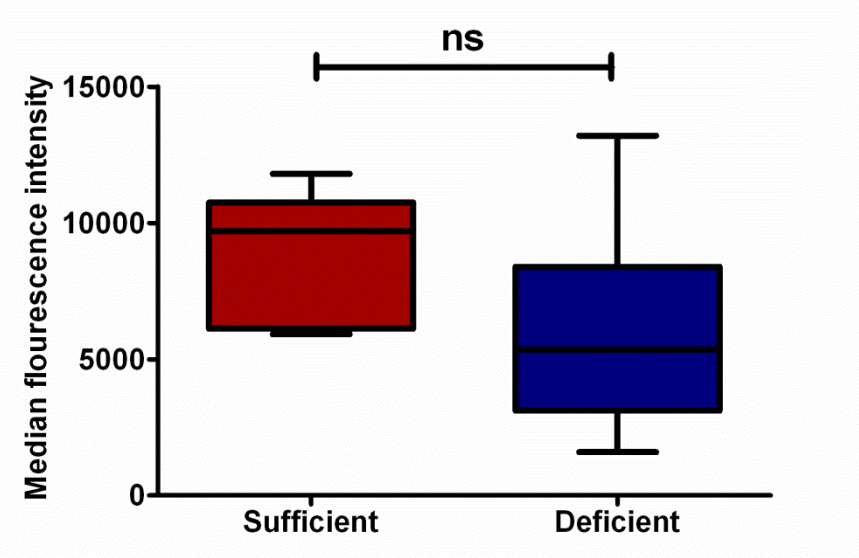

Figure 17 Vitamin C status (sufficient/deficient) does not alter neutrophils' reactive oxygen species production that is essential for its normal immunological function. Deficient neutrophils trended lower but there was no statistically significant difference (n= 8/group).

Chapter 3: Resolution of Sterile Inflammation: Role for Vitamin C

3.1 Introduction

Resolution of inflammation typically follows an ordered series of events orchestrated by different cell types.(89) During the early stages of inflammation, leukocytes such as polymorphonuclear neutrophils (PMN) are the first immune cells to arrive at the site of inflammation. PMNs are recruited by gradients of proinflammatory signals and usually reach peak numbers within 24–48 hrs. PMNs have short half-lives and are normally cleared from sites of inflammation by undergoing apoptosis.(90) Mobilized monocyte-derived macrophages extravasate to inflammatory tissue sites and clear apoptotic PMN in a non-phlogistic fashion by the process of efferocytosis. Apoptotic PMN release “find-me” signals that are sensed by extravasated macrophages.(91) Following phagocytosis, apoptotic PMN provides resolution cues to macrophages by evoking distinct signaling events that block release of proinflammatory mediators thus allowing further engulfment of apoptotic cells. Mantovani et al. and Fleming and Mosser note that mobilized macrophages are divided into three groups based upon their activation states.(92, 93) These include the M1, M2, and the recently described regulatory macrophages (Mres). M1 macrophages, classically referred to as activated macrophages, secrete proinflammatory factors that mediate host defense against invading pathogens. M2 macrophages, termed alternatively activated macrophages, are considered to be anti-inflammatory.(94, 95) Finally, Mres macrophages secrete considerable amounts of anti-inflammatory cytokines that prevent inflammatory and autoimmune pathology.(96, 97) Mres macrophages also secrete various lipid mediators that play critical roles in resolution of inflammation (see below).

Extensive new research has identified expression markers or phenotypic signatures for the various macrophage activation states in mice. They include gene expression changes in IL- 1β, TNFα, and iNOS for classical activation and arginase-1 (Arg1), chitinase 3-like 3 (YM-1), and TGFβ for the alternatively activated macrophages.(95)

Resolution of inflammation and restoration of normal tissue function prevent the development of “complications” of excessive inflammation, a process referred to as catabasis.(98) Catabasis is driven by synthesis and release of proresolution lipid mediators such as resolvins, protectins, and lipoxins.(99) Lipoxins and protectins are synthesized by lipoxygenase enzymes (such as 15-lipoxygenase (15-Lox)).(100, 101) Resolvins are derived from omega-3 polyunsaturated fatty acids such as docosahexaenoic acid and eicosapentaenoic acid.(102) They are products of metabolism involving 15-Lox and cyclooxygenase. Russell and Schwarze have reviewed the proresolution effects of proresolution mediators in a variety of inflammatory states.(103) However, their regulation by vitamin C (VitC, ascorbic acid, AscA) has yet to be examined.

VitC readily functions as one or two electron-reducing agents for many oxidants and serves as a primary chemical antioxidant in most cell types. It modulates complex biochemical pathways that form an essential part of normal metabolism of immune cells.(104) Intracellular levels of VitC in cells differ significantly from circulating plasma levels. In particular, VitC accumulates in millimolar quantities in immune cells such as PMN and macrophages in which intracellular VitC concentrations are typically 40–60 fold higher than that present in circulation.(27, 105) In PMN, Vissers and Wilkie showed that intracellular VitC levels regulate neutrophil apoptosis.(54) Further, VitC contributes

to the antioxidant defenses as well as normal PMN and macrophage function. Oberritter and colleagues showed that intracellular concentrations of VitC in macrophages are in the low millimolar range in freshly prepared peritoneal macrophages and *in vivo* or *in vitro* activation of peritoneal macrophages results in a significant decline in their VitC content.(106) Li et al. found that VitC deficiency worsens the inflammatory response following infection with the influenza virus.(107) Moreover, mice deficient in VitC generate excessive proinflammatory responses upon infection with the virulent bacterium *Klebsiella pneumonia*.(108) In humans, VitC levels are significantly reduced in critically ill patients and specifically in patients with poorly resolving proinflammatory states (e.g., sepsis, systemic inflammatory response syndrome).(22, 109) Several studies performed in septic patients have found that plasma VitC levels correlate inversely with the incidence of organ failure and directly with survival.(110, 111) We recently showed that VitC attenuates inflammation and normalizes PMN function in septic mice.(31, 112) We further showed that parenteral VitC attenuates proinflammatory biomarkers and reduces mortality in human sepsis.(32) However information is limited regarding the mechanism by which VitC regulates the progression and eventual resolution of inflammatory states.

In the current study we examined the progression and resolution of inflammation using a murine thioglycollate (TG)-elicited peritonitis model in VitC sufficient and deficient mice. While humans lack L-gulono-γ-lactone oxidase (Gulo), the final enzyme in the biosynthesis pathway of VitC,(55) mice express functional Gulo, resulting in cells and tissues generally maintaining high levels of VitC thereby complicating the translatability of VitC studies in mice, to humans. In order to establish the studies more

relevant to humans, TG-induced peritonitis was performed in transgenic mice lacking Gulo (Gulo−/−). Our studies reveal that progression and resolution of TG-induced inflammation is significantly delayed in VitC *deficient* mice. In particular, the spatiotemporal profile of pro- and anti-inflammatory mediator production by TG-elicited macrophages was significantly different between the VitC *sufficient* and *deficient* mice. Further, macrophage function and phenotype, as well as the antioxidant capacity of VitC *deficient* macrophages, was significantly impaired by the decline in intracellular VitC levels. Infusion of parenteral VitC as ascorbic acid (AscA) partly restored macrophage phenotype and function in VitC deficient mice.

3.2 Materials and Methods

3.2.1 Animals.

Gulo−/− mice were bred in-house from an established homozygous colony as previously described.(31) In order to maintain their plasma VitC levels similar to that observed in humans, VitC sufficient mice were fed ad libitum with regular chow and water supplemented with vitamin C (0.33 g/L) renewed twice per week. Gulo−/− mice were made VitC deficient by reducing VitC supplementation (0.033 g/L) for 1 week, followed by complete removal of dietary VitC for additional 2 weeks. We and others have shown that this reduced supplementation significantly decreases the concentration of VitC in immune cells, plasma, and organs.(31, 58, 59)

3.2.2 Thioglycollate Induced Peritonitis and Isolation of Mouse Peritoneal Macrophages.

Thioglycollate-mediated peritonitis was established by intraperitoneal (i.p.) injection of 1mL aged, sterile 3% TG solution to 9–11-week old Gulo−/− mice. Thirty minutes

following i.p. challenge, some VitC deficient mice were randomized to receive daily i.p. injection of VitC as AscA (200mg/kg in saline) for a further 3 or 5 days. Untreated mice received i.p. saline instead of VitC. Mice were euthanized on day 3 or day 5, and the peritoneal cavity lavaged with 7mL of Hanks' balanced salt solution (HBSS) containing 1% bovine serum albumin (BSA). The lavage was centrifuged and the resulting leukocyte pellet was washed with HBSS and resuspended in RPMI-1640 medium. Cell counts of the peritoneal lavage were performed using a Hemocytometer. Cytochemical staining of peritoneal cells was performed using HARLECO Hemacolor solution (EMDMillipore).(112) PMNs were then separated from macrophages by adherence to a plastic dish as described previously.(60, 112) Peritoneal macrophages were plated at a density of 2×10^6 cells in 35mm dishes in growth media (DMEM, 10% FBS). Media were changed after 2 h to remove floating cells prior to experimentation.

3.2.3 Cell Culture.

Human acute monocytic leukemia suspension cell line (THP-1) was obtained from ATCC (Manassas, VA). THP-1 cells were maintained in RPMI-1640 medium containing 10% FBS according to the instructions supplied. For induction of macrophages, PMA (100 nM) was added to the medium and cells were seeded at a density of 0.1×10^6 cells/cm2 into tissue culture dishes and maintained in a humidified atmosphere of 95% air and 5% CO2. Media containing PMA were replaced every 2 days, and experiments started after 5 days in culture, when the cells were phenotypically macrophage.(113)

3.2.4 Vitamin C Analysis.

Plasma and intracellular VitC levels of peritoneal macrophages seeded onto 35mm dishes were measured using a fluorescence end-point assay adopted from Vislisel et

al..(114) Plasma was deproteinized as described previously.(32) Briefly, 0.2mL of cold 20% trichloroacetic acid (TCA) and 0.2mL of cold 0.2% dithiothreitol (DTT) were added to 0.1mL of plasma, vortexed for 2min, and centrifuged (10,000 g, 10 min, 4◦C). Supernatants were aliquoted and frozen at −70◦C for batch analysis. Peritoneal macrophages were similarly extracted with TCA and DTT and frozen at −70◦C for batch analysis. Supernatant or AscA standards were transferred in triplicate to a 96-well plate. Assay buffer containing 1M sodium acetate, pH 5.5, and 1mM TEMPOL was added to each well and the plate was incubated for 10 minutes at room temperature. Freshly prepared o-phenylenediamine (OPDA) solution (5.5mMOPDA in acetate buffer of pH 5.5) was then added. After further 30min incubation in the dark, fluorescence was measured at an emission wavelength of 425 nm following excitation at 345 nmand values determined after comparison to a standard curve. Intracellular AscA levels were estimated spectrophotometrically from the standard curve and the intracellular concentrations derived from the measured amount of AscA and the knownmacrophage cell volume.(115)

3.2.5 RNA Isolation and Real-Time Quantitative PCR (QPCR) Analysis.

Isolation of total RNA and real-time QPCR analyses were performed as described previously.(30) Briefly total RNA was extracted and purified using QIAshredders and RNeasy columns according to the manufacturer's specifications (Qiagen). Total RNA (1μg) was reverse-transcribed into cDNA using the High Capacity cDNA Reverse Transcription kit. Complimentary DNA (cDNA) was diluted (1 : 500) and real time QPCR performed using POWER SYBR Green QPCR Master Mix. Primers were designed to anneal to sequences on separate exons or to span two exons. Primers used for QPCR

are listed in Table 1. Cycling parameters were 95^0C, 10min, 40 cycles of 95^0C, 15 sec, and 60^0C, 1min. A dissociation profile was generated after each run to verify specificity of amplification. All PCR assays were performed in triplicate. No template controls and no reverse transcriptase controls were included. The mRNA expression in macrophages from a "sufficient" mouse or a media well was set to "1." The mRNA expression of all other samples was compared relative to this sample which was used as the baseline. 18S rRNA was used as housekeeping gene against which all the samples were normalized for differences in the amount of total RNA added to each cDNA reaction and for variation in the reverse transcriptase efficiency among the different cDNA reactions. Automated gene expression analysis was performed using the Comparative Quantitation module of MxPro QPCR Software (Agilent).

Table 1 Primer sequences for Real Time Quantitative PCR.

Name	Sequence 5' to 3'
Murine IL-1β forward	CTGAACTCAACTGTGAAATGCC
Murine IL-1 β reverse	CAGGTCAAAGGTTTGGAAGC
Murine TNF-α forward	GATGAGAAGTTCCCAAATGGC
Murine TNF- α reverse	TTGGTGGTTTGCTACGACG
Murine MCP-1 forward	TTCTGGGCCTGCTGTTCACAG
Murine MCP-1 reverse	CCAGCCTACTCATTGGGATCATCTTGC
Murine YM1 forward	CAAGACTTGCGTGACTATGAAGC
Murine YM1 reverse	AGGTCCAAACTTCCATCCTCC
Murine Arg1 forward	AGGAAAGCTGGTCTGCTGG
Murine Arg1 reverse	TTGAAAGGAGCTGTCATTAGGG
Murine IL-10 forward	CAAGGAGCATTTGAATTCCC
Murine IL-10 reverse	ATTCATGGCCTTGTAGACACC

Murine Gal1 forward	CAGCAACCTGAATCTCAAACC
Murine Gal1 reverse	AGTGTAGGCACAGGTTGTTGC
Murine 15-Lox forward	TGGTGGCTGAGGTCTTTGC
Murine 15-Lox reverse	TCTCTGAGATCAGGTCGCTCC
Human IL-6 forward	GGATTCAATGAGGAGACTTGCC
Human IL-6 reverse	TCTGCAGGAACTGGATCAGG
Human IL-8 forward	GTGTGAAGGTGCAGTTTTGC
Human IL-8 reverse	GAGCTCTCTTCCATCAGAAAGC
Human TNF-α forward	CCTCTTCTCCTTCCTGATCG
Human TNF- α reverse	CGAGAAGATGATCTGACTGCC

3.2.6 Western Blot Analysis.

Mouse macrophage and THPI whole-cell extracts were isolated for western blot analysis as described previously.(30) Proteins were resolved by SDS polyacrylamide gel electrophoresis (4–20%) and electrophoretically transferred to polyvinylidene fluoride membranes (0.2 μm pore size). Immunodetection was performed using chemiluminescent detection with the Renaissance Western Blot Chemiluminescence Reagent Plus (Perkin Elmer Life Sciences Inc., Boston, MA). Blots were stripped using the Restore Western Blot Stripping Buffer (Pierce Biotechnology Inc., Rockford, IL) as described by the manufacturer. Purified rabbit polyclonal antibodies to phospho-NFκB p65 (Ser276, Cell Signaling), NFκB p65 (sc-109, Santa Cruz Biotechnology), iNOS (sc-650, Santa Cruz Biotechnology), and actin (sc-1616, Santa Cruz Biotechnology) were used.Optical densities of antibody-specific bands were determined using Quantity One acquisition and analysis software (Bio-Rad, Hercules, CA).

3.2.7 Flow Cytometry.

Mouse peritoneal lavage obtained on day 3 and day 5 from VitC sufficient or deficient mice following induction of TG-induced peritonitis was pelleted by centrifugation at 4^0C. Cells were resuspended in FACS buffer containing Fc receptor block (CD16/CD32 eBioscience) for 10min at 4^0C. Aliquots of the suspension were incubated at 4^0C for 30min (in the dark) with fluorescein isothiocyanate (FITC-) conjugated anti-mouse CD45 (eBioscience) and allophycocyanin (APC-) conjugated anti-mouse CD11b (eBioscience). Unstained and single color controls were employed for each experiment. Samples were then fixed with 1% formaldehyde for 20min at room temperature. All runs were performed on a BD Accuri C6 Flow Cytometer (BD Accuri Cytometers, MI, USA) and analyzed using FlowJo software (Tree Star, Ashland, OR).

3.2.8 Fluorescence Microscopy.

Fluorescence microscopy for evaluation of mitochondrial reactive oxygen species (ROS) in macrophages was performed using the cell-permeant probe MitoTracker Red CMXRos as described by the manufacturer. Briefly, macrophages from VitC sufficient or deficient mice were grown on Ibidi 6-channel IbiTreat μ-slide VI. Following treatments (H2O2, 18 hours) culture media were aspirated and cells were fixed in 3.7% paraformaldehyde in PBS for 10 minutes at 4^0C. Fluorescence imaging was performed using an Olympus model IX70 inverted phase microscope (Olympus America, Melville, NY) outfitted with an IX-FLA fluorescence observation system equipped with a TRITC filter cube (530–560nmexcitationand590–650nmemission, Chroma Technology Corp. Brattleboro, VT) through an Uplan FI objective (40x). Fluorescence images were digitized and captured by a MagnaFire digital camera (Optronics, Goleta, CA).

3.2.9 Lipid Extraction and Analysis.

Quantitative analysis of eicosanoids was performed as previously described by us with minor modifications.(116-121) Briefly, peritoneal lavage was clarified by centrifugation and 0.05% BHT and 10 ng of each internal standard added. The internal standards used were ($d4$) 8-iso PGF2α, ($d11$) 5-iso PGF2α-VI, ($d4$) 6k PGF1α, ($d4$) PGF2α, ($d4$) PGE2, ($d4$) PGD2, ($d4$) LTB4, ($d5$) Lipoxin A4, ($d5$) Resolvin D2, ($d4$) TXB2, ($d4$) LTC4, ($d5$) LTD4, ($d5$) LTE4, ($d8$) 5-hydroxyeicosatetranoic acid (5HETE), ($d8$) 15-hydroxyeicosatetranoic acid (15HETE), ($d8$) 14,15 epoxyeicosatrienoic acid, ($d8$) arachidonic acid, and ($d5$) eicosapentaenoic acid. The samples were mixed by vortexing and subjected to purification via solid phase extraction (SPE) using a 24 port vacuum manifold (Sigma-Aldrich). Strata-X SPE columns (Phenomenex) were washed with methanol (2 mL) and then dH2O (2mL). The samples were applied to the column. Thereafter the sample vials were rinsed with 5% MeOH (2mL), which was then used to wash the columns. Finally, the eicosanoids were eluted with isopropanol (2 mL). The eluents were then dried under vacuum and reconstituted in LCMS grade 50 : 50 EtOH: dH2O (100μL) for eicosanoid quantitation via UPLC ESI-MS/MS analysis. A 14-minute reversed-phase LC method utilizing a Kinetex C18 column (100 × 2.1 mm, 1.7 μm) and a Shimadzu UPLC was used to separate the eicosanoids at a flow rate of 500 μL/min at 50 ^{0}C. The column was first equilibrated with 100% Solvent A (acetonitrile : water : formic acid (20 : 80 : 0.02, v/v/v)) for two minutes and then 10 μL of sample was injected. 100% Solvent A was used for the first two minutes of elution. Solvent B (acetonitrile : isopropanol (20 : 80, v/v)) was increased in a linear gradient to 25% Solvent B to 3 minutes, to 30% by 6 minutes, to 55% by 6.1 minutes, to 70% by 10 minutes, and to 100% by 10.1 minutes. 100% Solvent B was held until 13 minutes and

then was decreased to 0% by 13.1 minutes and held at 0% until 14 minutes. The eluting eicosanoids were analyzed using a hybrid triple quadrapole linear ion trap mass analyzer (ABSciex 6500 QTRAP) via multiple-reaction monitoring in negative-ion mode. Eicosanoids were monitored using species specific precursor → product MRM pairs. The mass spectrometer parameters used were curtain gas: 30; CAD: High; ion spray voltage: −3500V; temperature: 300∘C; Gas 1: 40; and Gas 2: 60; declustering potential, collision energy, and cell exit potential were optimized per transition.

3.2.10 Statistical Analysis.

Statistical analysis was performed using SAS 9.3 and GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). Data are expressed as mean ± SE. Results were compared using one-way ANOVA and the post-hoc Tukey test. Statistical significance was confirmed at a *p*-value of <0.05.

3.3 Results

3.3.1 VitC Deficiency Alters the Progression of TG-Induced Peritoneal Inflammation.

In order to make the Gulo−/− mice VitC deficient, supplementation of water with AscA was withdrawn as described in the Methods section. Within 3 weeks of removal of VitC supplementation, plasma VitC levels of Gulo−/− mice declined significantly (Figure 1). This decline was not associated with deleterious changes in weight or health status in the VitC deficient mice (data not shown).

To determine whether VitC deficiency impacts the progression of peritoneal inflammation, VitC sufficient or deficient mice were injected with TG and the progression of inflammation was monitored on days 3 and 5 (as described in Section 2). Some VitC

deficient mice were injected i.p. with AscA (200mg/kg) prior to harvest of peritoneal lavage (see Section 2). Daily i.p. administration of ascorbate for 3 days restored circulating plasma VitC concentrations in these mice to levels observed in the VitC sufficient mice (Figure 18). In all 3 groups, the infiltration of inflammatory cells on day 1 was similar to that observed in wild type mice and was in agreement with our previous observations (Table 2).(112, 122) As seen in Table 2, there was also no difference in the total number of cells elicited from the peritoneal exudation of day 3 and day 5. However, significant differences in the cellular composition of the lavage were evident on day 3 and day 5 between the 3 groups. In the VitC sufficient mice group, mononuclear cells were the predominant cell type on days 3 and 5 (Table 2). PMN numbers, which peaked on day 1,(112) returned to baseline by days 3 and 5. In contrast, significantly elevated numbers of PMNs persisted in the peritoneal exudates of VitC deficient mice on days 3 and 5 (Table 2). Infusion of AscA reduced PMN numbers by day 3 with a significant decline in PMN numbers to baseline similar to the VitC sufficient mice by day 5 Table 2.

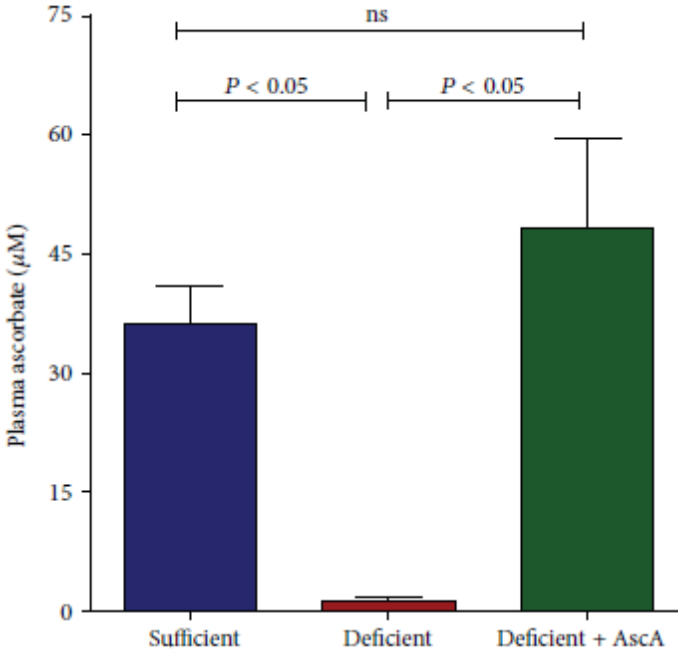

Figure 18 Vitamin C deficiency alters the progression of TG-induced peritoneal inflammation. Plasma VitC levels were measured in VitC *sufficient* and *deficient* Gulo -/− mice as well as in *deficient* mice treated daily with i.p. AscA for 3 days (*N*= 3–6 mice/group, ns = not significant).

Table 2: Differential cell counts from peritoneal exudates following thioglycollate-induced peritonitis (N = 6–8 mice/group, n.d. = not determined).

		Sufficient	Deficient	Deficient + AscA
Day 1	PMN (x10^6)	20.02 ± 3.4	23.2 ± 2.6	n.d.
	M0 (x10^6)	5.0 ± 0.8	3.9 ± 0.6	n.d.
Day 3	PMN (x10^6)	1.8 ± 0.4	8.1 ± 1.9[a]	3.8 ± 0.4
	M0 (x10^6)	18.4 ± 3.2	13.1 ± 3.1	14.5 ± 3.2
Day 5	PMN (x10^6)	1.0 ± 0.6	4.4 ± 1.1[b]	0.4 ± 0.2[c]
	M0 (x10^6)	18.8 ± 5.6	22.1 ± 4.2	14.3 ± 4.1

[a] Sufficient versus deficient, P = 0.006.
[b] Sufficient versus deficient, P = 0.02.
[c] Deficient versus deficient + AscA, P = 0.02.

3.3.2 Spatiotemporal Profiling of Inflammatory Mediators following TG-Induced Peritoneal Inflammation.

We previously observed that TG-elicited PMN from VitC deficient mice (on day 1) demonstrated increased expression of the proinflammatory genes TNFα and IL-1β.(112) Here we examined the expression of multiple pro- and anti-inflammatory mediators originating from macrophages, the predominant cell type recruited to the inflamed peritoneum on days 3 and 5. As seen in Figure 19, significant differences were evident in the magnitude of pro- and anti-inflammatory mediator expression on days 3 and 5. On day 3, increased expression of the pro-inflammatory mediators (IL-1β, TNFα, and MCP-1) was observed in macrophages from VitC deficient mice when compared to

macrophages from VitC sufficient mice (Figure 19(a), (A), (C), and (E)).

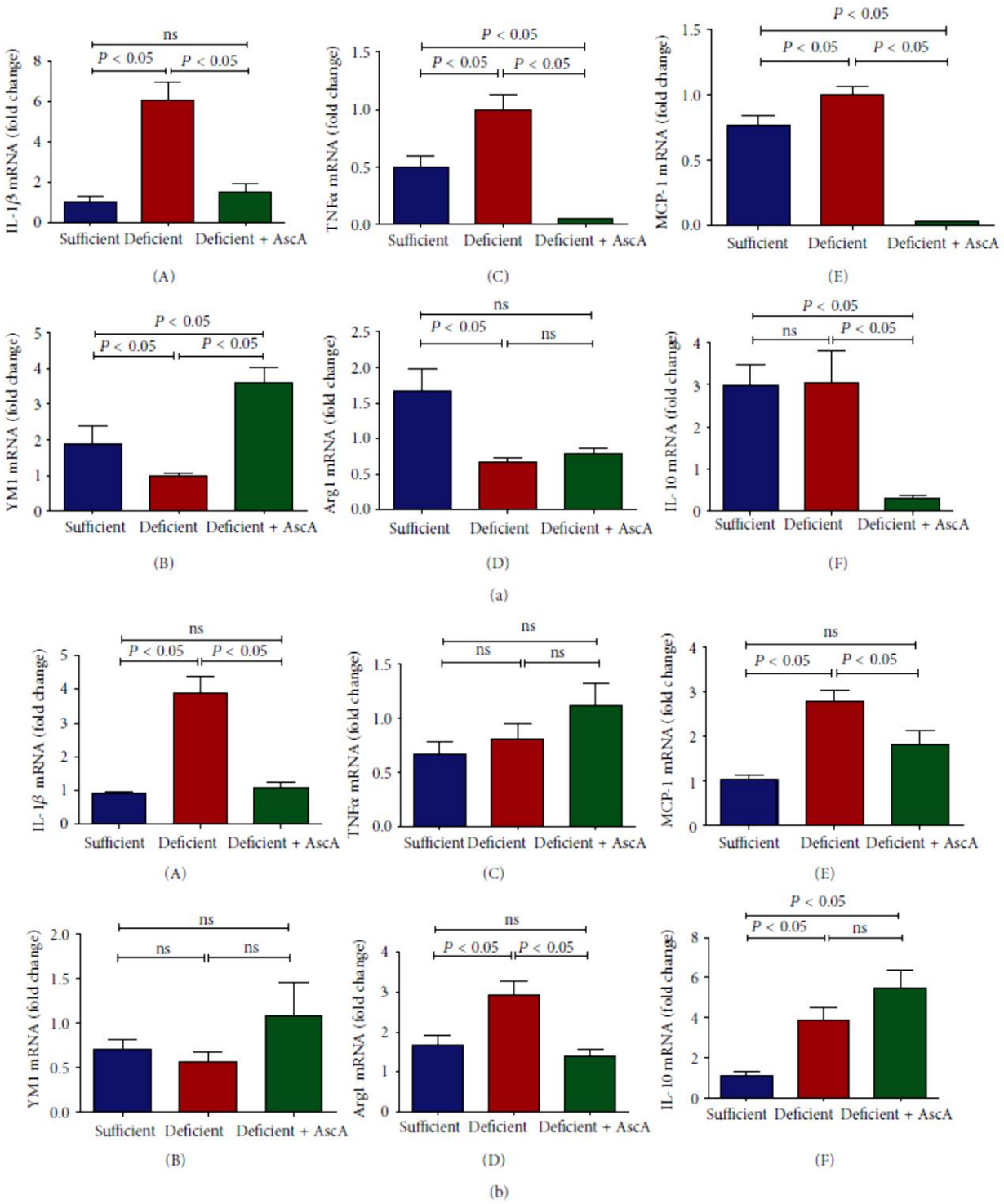

Figure 19 Spatiotemporal profiling of inflammatory mediators following TG-induced peritoneal inflammation. Real time QPCR for IL-1β, TNFα,MCP-1, YM1, Arg1, and IL-10mRNA from peritoneal macrophages elicited on day 3 (a) and day 5 (b) following TG-induced peritonitis from VitC sufficient and deficient Gulo−/− mice. Following TG challenge, some VitC deficient mice were randomized to receive daily i.p. injection of VitC as

AscA (200mg/kg in saline) for a further 3 days (day 3, deficient + AscA) or 5 days (day 5, deficient + AscA) (*N* = 6 mice/group, ns = not significant)

Pro-inflammatory gene expression was significantly attenuated by i.p. infusion of AscA in the VitC deficient mice (Figure 19(a), (A), (C), and (E)). In contrast, anti-inflammatory gene expression (YM1 and Arg1, but not IL-10) was elevated in macrophages from VitC sufficient mice (Figure 19(a), (B), (D), and (F)). Daily AscA infusion induced YM1 expression in VitC deficient macrophages but failed to restore Arg1 expression. IL-10 expression on the other hand was significantly lowered by AscA infusion on day 3 (Figure 19(a), (B), (D), and (F)). On day 5 (Figure 19(b)), pro-inflammatory gene expression remained persistently elevated in macrophages from VitC deficient mice (IL-1β andMCP-1) but was attenuated by AscA infusion. In contrast, anti-inflammatory gene expression in VitC deficient macrophages was significantly higher when compared to macrophages from VitC sufficient mice (Arg1, IL-10). AscA infusion did not alter anti-inflammatory gene expression on day 5 although Arg1 levels were now similar to that observed in VitC sufficient mice (Figure 19(b), (B), (D), and (F)).

3.3.3 Ex Vivo Bacterial Lipopolysaccharide Differentially Activate Pro-inflammatory Gene Expression in Macrophages from VitC Sufficient and Deficient Mice.

Canali et al. recently showed that in contrast with baseline physiological activation, exposure to a second “hit” such as an inflammatory stimulus results in a markedly different modulation of gene expression in human peripheral blood mononuclear cells in the presence or absence of VitC supplementation.(123) To examine whether peritoneal macrophages would exhibit an altered modulation of gene expression, we exposed day 3 peritoneal macrophages from VitC sufficient and deficient mice to bacterial

lipopolysaccharide (LPS, 50 ng/mL). Some macrophages were incubated with AscA (3 mM, 16 hours) prior to LPS exposure. As seen in Figures 20(a) and 20(b), LPS exposure

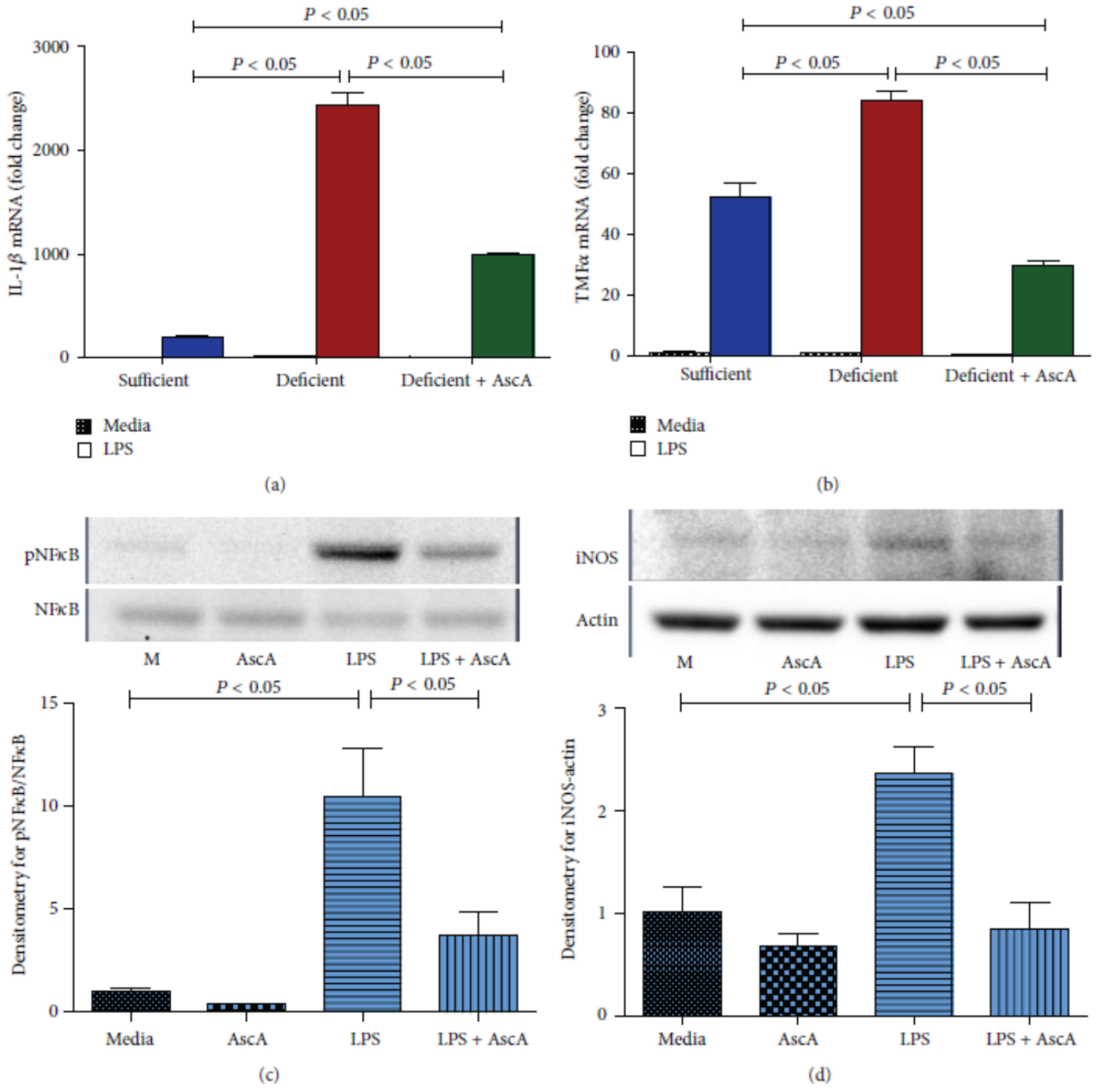

Figure 20 LPS differentially activates pro-inflammatory gene expression in macrophages from vitamin C sufficient and deficient mice. Peritoneal macrophages elicited on day 3 following TG-induced peritonitis from VitC sufficient and deficient Gulo−/− mice were exposed to LPS (50 ng/mL) for 4 hours. Macrophages from some VitC deficient mice were incubated with AscA (3 mM, 16 hours) prior to LPS exposure (deficient + AscA). Real time QPCR was performed for IL-1β (a) and TNFα (b) (N = 6/group). (c) Upper panel: representative western blot for expression of phospho-NFκB andNFκB from VitC deficient macrophages exposed to media alone (M), AscA (3 mM, 16 hours (AscA)), LPS (50 ng/mL) for 1 hour (LPS), or AscA for 16

hours followed by LPS for 1 hour (LPS + AscA). Lower panel: densitometry for normalized expression of phospho-NFκB from macrophages (N = 3/group). (d) Upper panel: representative western blot for expression of iNOS and actin from macrophages groups described in (c) and exposure to LPS (50 ng/mL) for 4 hour. Lower panel: densitometry for normalized expression of iNOS from macrophages (N = 3/group).

resulted in a robust increase in expression of pro-inflammatory markers (IL-1β, TNFα) in macrophages from VitC sufficient mice. Pro-inflammatory gene expression was also induced in macrophages from VitC deficient mice, but the magnitude of induction was significantly greater than that observed in the VitC sufficient macrophages (Figures 20(a) and 20(b)). Importantly, exposure of VitC deficient macrophages to AscA prior to LPS significantly attenuated IL-1β and TNFα expression. Increased NFκB activation (Figure 20(c)) and iNOS protein expression (Figure 20(d)) was observed upon exposure of VitC deficient macrophages to LPS (P < 0.05). AscA pretreatment attenuated NFκB activation and iNOS expression in VitC deficient macrophages.

3.3.4 VitC Regulates Macrophage Function during the Resolution of Inflammation.

Macrophages undergo reprogramming to adopt a variety of functional phenotypes upon receiving differentiation cues from their surrounding environment.(124) It was recently shown that macrophage reprogramming is vital for the resolution of acute inflammation.(125) We examined whether macrophage VitC sufficiency or deficiency could influence macrophage function during resolution of acute inflammation. Macrophages were isolated on day 3 following TG-mediated peritonitis from VitC sufficient or deficient mice and intracellular concentrations of VitC measured (as described in Section 2). Some VitC deficient mice were injected daily with i.p. AscA (200mg/kg) prior to harvest of peritoneal lavage (see Section 2).

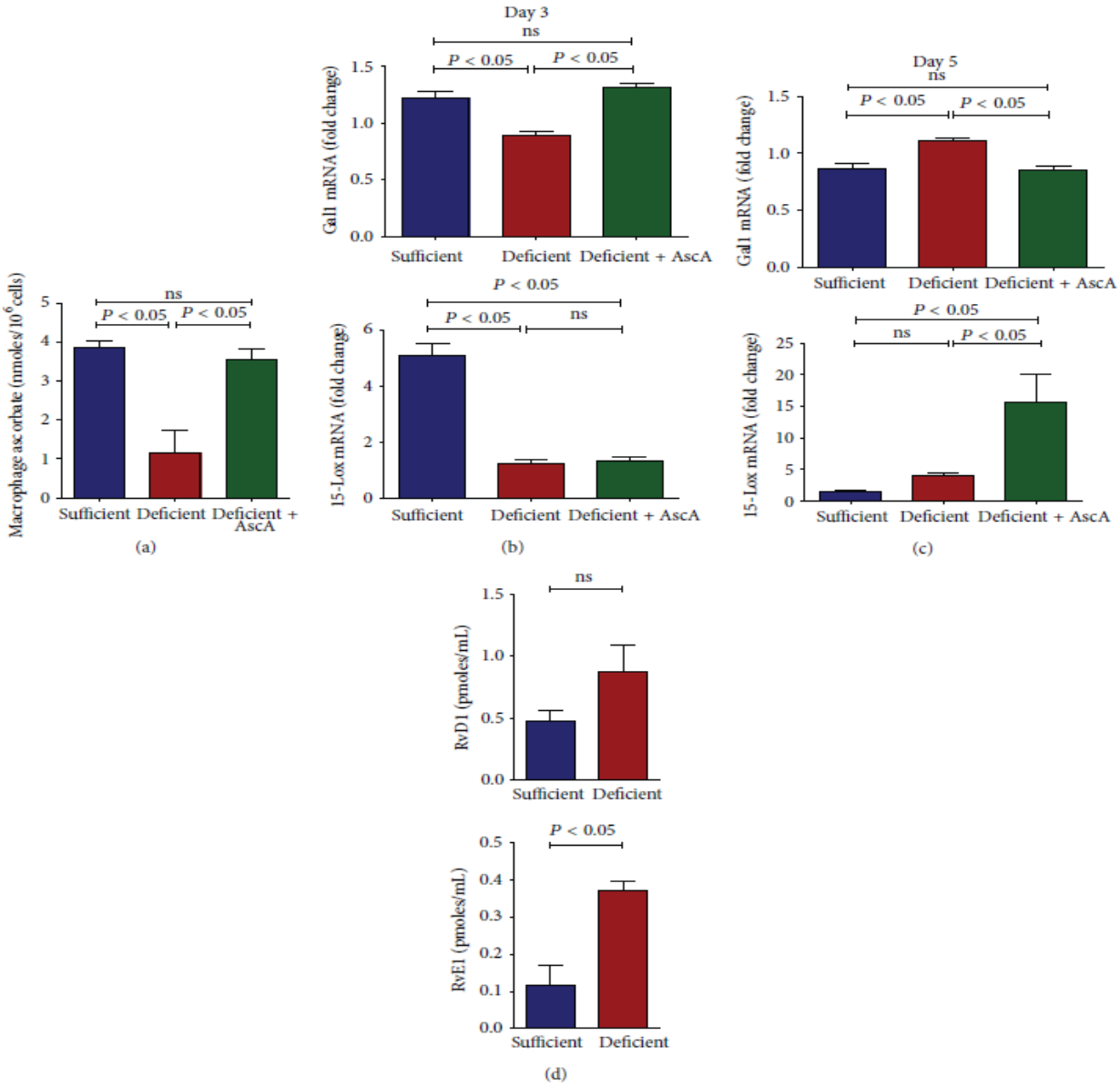

Figure 21 Vitamin C regulates macrophage function during the resolution of inflammation. (a)Macrophages were isolated on day 3 following TG-mediated peritonitis from VitC sufficient or deficient mice as well as in deficient mice treated daily with i.p. AscA for 3 days and intracellular concentrations of VitC measured (*N* = 3–10 mice/group, ns = not significant). (b) Real time QPCR for Gal1 and 15-Lox from peritoneal macrophages elicited on day 3 following TG-induced peritonitis from VitC sufficient and deficient Gulo−/− mice. Thirty minutes following TG challenge, some VitC deficient mice were randomized to receive i.p. injection of VitC as AscA (200mg/kg in saline) for a further 3 days (deficient + AscA). (*N* = 6 mice/group, ns = not significant). (c) Real time QPCR for Gal1 and 15-Lox from peritoneal macrophages elicited on day 5 following TG-induced peritonitis from VitC sufficient and deficient Gulo−/− mice. Thirty minutes following TG challenge, some VitC deficient mice were randomized to receive i.p. injection of VitC as AscA (200mg/kg in saline) for a further 5 days (Deficient + AscA) (*N* = 6 mice/group, ns = not significant). (d) UPLC ESI-MS/MS quantification of resolvin D1 (RvD1) and E1 (RvE1) in peritoneal lavage on day 5 following TG-induced peritonitis from VitC sufficient and deficient Gulo−/− mice (*N*= 3-4 mice/group, ns = not significant).

As seen in Figure 21(a), macrophages from VitC sufficient mice have high intracellular

VitC concentrations. In contrast, intracellular ascorbate levels were significantly depleted in macrophages from VitC deficient mice. Daily i.p. administration of AscA for 3 days also restored macrophage intracellular concentrations to levels observed in the VitC sufficient mice (Figure 21(a)). Gal-1 and 15-Lox expression is induced in macrophages during peritonitis. Their expression is associated with generation of pro-resolving lipid mediators and successful resolution of inflammation.(99, 126) Therefore we examined Gal-1 and 15-Lox expression in day 3 and day 5 macrophages from VitC sufficient or deficient mice. As seen in Figure 21(b), Gal-1 and 15-Lox expression was significantly induced in macrophages from VitC sufficient mice on day 3 when compared to macrophages from VitC deficient mice. AscA infusion restored Gal-1 expression in VitC deficient macrophages but did not affect 15-Lox expression on day 3 (Figure 21(b)). In contrast, Gal-1 expression in VitC deficient macrophages was delayed and observed to be higher on day 5 following TG-induced peritonitis (Figure 21(c)). 15-Lox expression was induced by AscA infusion on day 5 and was higher than that observed in macrophages from VitC sufficient or deficient mice. In agreement with the expression data seen above, resolvin (Figure 21(d)) production was higher on day 5 in VitC deficient mice indicating delayed resolution of inflammation.

3.3.5 VitC Influences Macrophage Phenotype during Resolution of Inflammation.

Rostoker et al. recently showed that Gal-1 was selectively expressed in CD11b-high macrophages, and its expression declined significantly once these cells converted toward a CD11b-low phenotype.(127) Moreover, CD11blow macrophages are the predominant subtype to depart the peritoneum.(127) To determine whether VitC regulated reprogramming of peritoneal macrophages to pro-resolution CD11b-low

phenotype we used flow cytometry to examine the distribution of CD11b-high and CD11b-low population on macrophages isolated on day 3 and day 5 following TG-induced peritonitis in VitC sufficient or deficient mice. As seen in Figure 22, there was a significant transition from CD11b-high to a CD11b-low phenotype observed from day 3 to day 5 in the VitC sufficient macrophages. This was not evident in the macrophages from VitC deficient mice indicative of a delay in the resolution of TG-induced peritonitis in these mice.

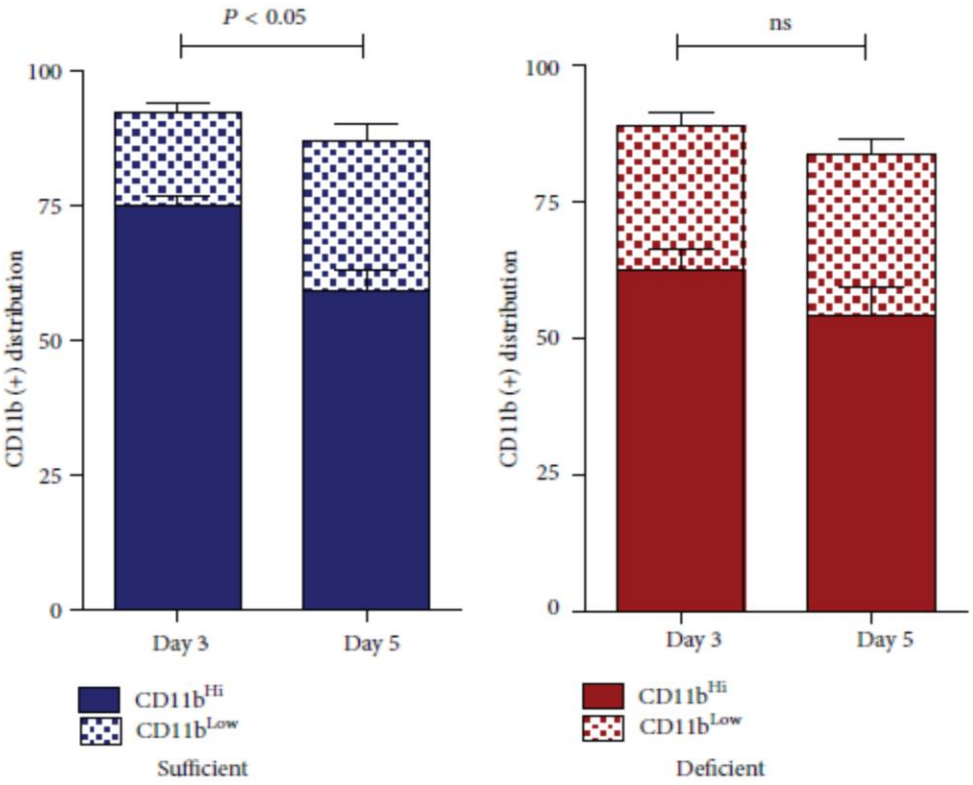

Figure 22 Vitamin C influences macrophage phenotype during resolution of inflammation. Flow cytometry for distribution of CD11bhigh and CD11blow population from macrophages isolated on day 3 and day 5 following TG-induced peritonitis in VitC sufficient or deficient mice (N = 5mice/group, P < 0.05, CD11blow day 3 versus day 5, ns = not significant).

3.3.6 Macrophages Deficient in VitC Have Reduced Antioxidant Capacity.

Activated macrophages potentially generate mitochondria-damaging deleterious reactive oxygen species (ROS).

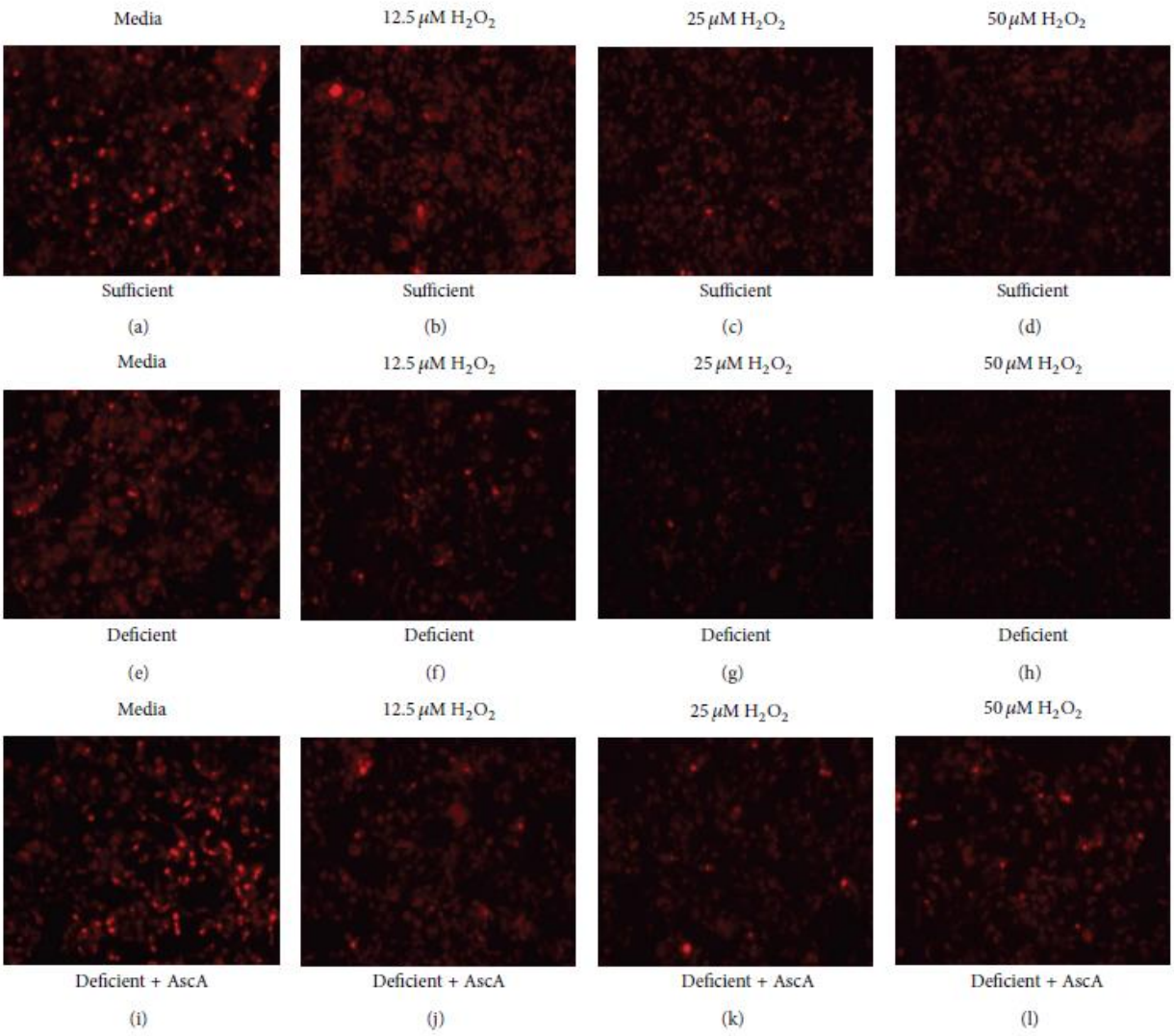

Figure 23 Macrophages deficient in Vitamin C have reduced antioxidant capacity. Peritoneal macrophages elicited on day 3 following TG-induced peritonitis fromVitC sufficient ((a)–(d)) and deficient Gulo−/− ((e)–(h)) mice were exposed to 12.5, 25, and 50 μMH2O2 for 18 hours and probed with MitoTracker Red CMXRos. Macrophages from some VitC deficient mice were incubated with AscA (3 mM, 16 hours) prior to exposure to H2O2 followed by staining with MitoTracker Red CMXRos (Deficient + AscA, ((i)–(l))).

Release of large amounts of ROS during activation exposes macrophages themselves to oxidant stresses not encountered by most other cell types.(128) To test whether VitC deficiency affected mitochondrial function in macrophages, we exposed peritoneal

macrophages (day 3) from VitC sufficient or deficient mice to varying concentrations of H_2O_2 for 18 hours and stained the cells with Mito- Tracker Red CMXRos as described in Section 2. This probe is selectively retained by mitochondria, where it is oxidized to its fluorescent form. As seen in Figures 23(a) and 23(e), control macrophages from VitC sufficient or deficient mice were stained brightly with the probe. Oxidative stress from exposure to H_2O_2 decreased fluorescent staining in macrophages from both VitC sufficient and VitC deficient mice. However, the magnitude of decrease was significantly greater in macrophages from VitC deficient mice (Figures 23(f) – 23(h)). This decrease was partially reversed by pretreatment of VitC deficient macrophages with AscA (Figures 23(j) and 23(k)). These studies indicate that VitC deficient macrophages sustain greater mitochondrial dysfunction when challenged with ROS.

3.3.7 VitC Attenuates Proinflammatory Gene Expression in Human Monocyte/Macrophages.

To address whether the modulatory activities of VitC are effective in human monocyte/ macrophages, we exposed THP-1 cells to bacterial LPS and examined the mRNA expression of the pro-inflammatory genes IL-6, IL-8, and TNFα. Since the culture medium in which THP-1 cells are grown contains no VitC, we increased intracellular concentrations of VitC by loading cells with AscA prior exposure to LPS. As seen in Figure 24(a), exposure of THP-1 cells to LPS resulted in a robust activation of mRNA for IL-6, IL-8, and TNFα. Loading cells with AscA did not affect baseline pro-inflammatory gene expression. However LPS exposure of AscA loaded cells resulted in significant attenuation of mRNA expression of these proinflammatory genes.

Attenuation of mRNA expression was likely achieved by reduction in activation of the transcription factor NFκB following LPS exposure (Figure 24(b)).

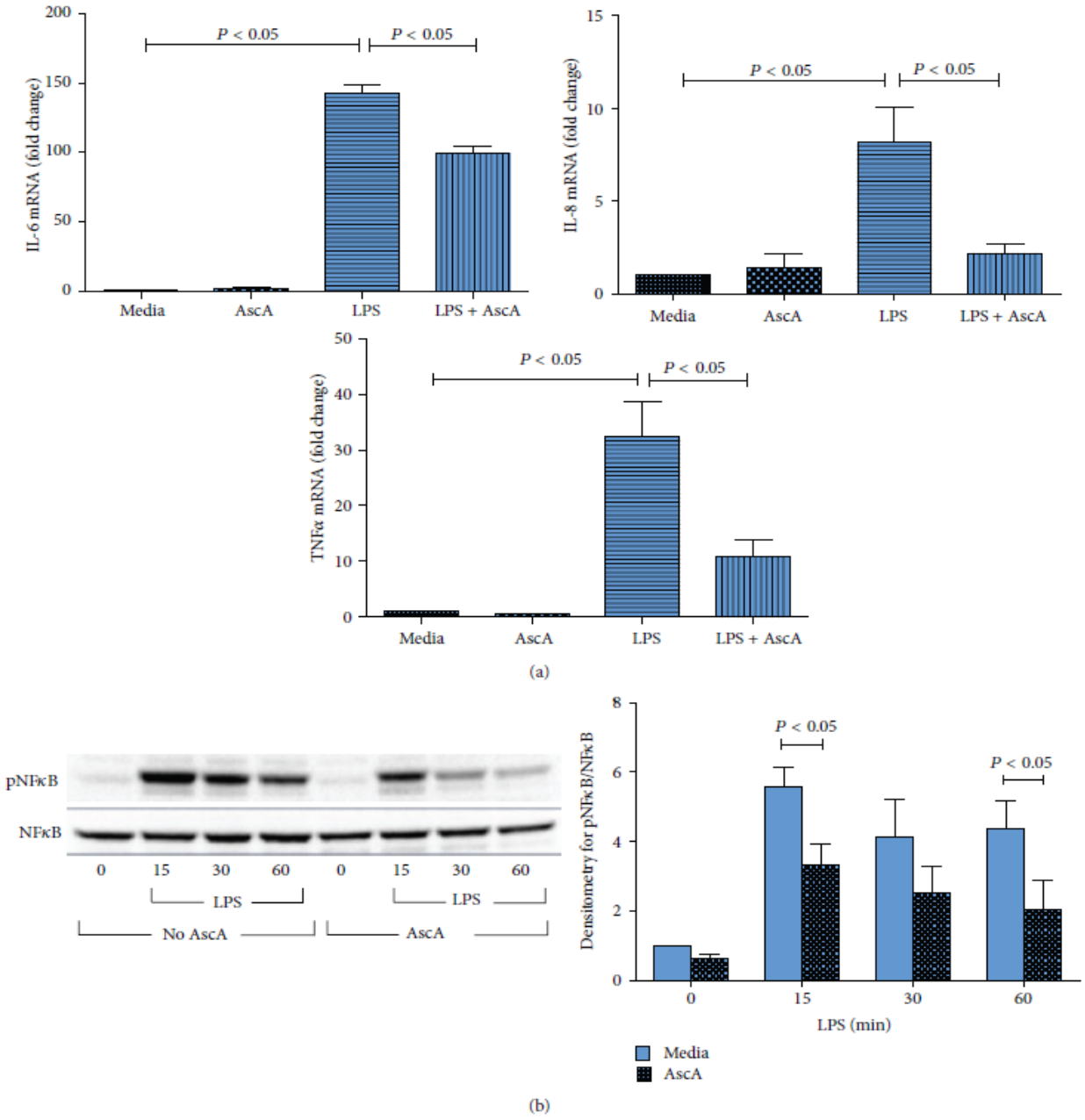

Figure 24 Vitamin C attenuates proinflammatory gene expression in human THP-1 monocyte/macrophages. THP-1 macrophages were exposed to media alone (Media), AscA (3mM, 16 hours (AscA)), and LPS (50 ng/mL) for 4 hour (LPS) or AscA for 16 hours followed by LPS for 4 hour (LPS + AscA). (a) Real time QPCR for IL-6, IL-8, and TNFα was performed as described in Section 2 (N = 4/group; P < 0.05, Media versus LPS and LPS versus LPS + AscA). (b) Left panel: representative western blot for expression of phospho-NFκB andNFκB from THP-1 groups described above following exposure to LPS (50 ng/mL) for 0, 15, 30, and 60 minutes. Right panel: densitometry for normalized expression of phospho-NFκB fromTHP-1 (N = 4/group, P < 0.05, LPS versus LPS + AscA).

3.4 Discussion

In this study, we examined the mechanism by which VitC regulates the resolution of sterile inflammation. Using mice lacking the ability to synthesize VitC, we showed that subnormal cellular VitC levels negatively impact the progression and resolution of sterile inflammation. In particular, our results demonstrate that low circulating VitC levels are associated with significant delays in the timing of resolution of inflammation. This apparent VitC-dependent process primarily occurs due to failure of macrophages to transition from a pro-inflammatory to a pro-resolving phenotype. The initial response to sterile inflammation was identical in VitC sufficient and deficient mice. During the early pro-inflammatory phase no differences in the cell numbers or cell types were observed. However, by days 3 and 5, VitC deficient mice exhibited significant numbers of PMN in peritoneal exudates (Table 2). Spatiotemporal mRNA profiling of macrophage-derived inflammatory mediators revealed dramatic differences in the magnitude of pro and anti-inflammatory mediator gene expression (Figure 19). Macrophages from VitC sufficient mice displayed prominent anti-inflammatory phenotypes, while VitC deficient macrophages persistently expressed mRNA for IL-1β, TNFα, and MCP-1, findings characteristic of a pro-inflammatory phenotype. LPS activation of day 3 macrophages from VitC deficient mice led to pro-inflammatory gene expression that was significantly greater in magnitude than that observed in VitC loaded macrophages (Figure 20). LPS stimulation was characterized by enhanced NFκB activation and iNOS induction in VitC deficient macrophages (Figure 20). Importantly, on day 3, VitC sufficient macrophages demonstrated cues for reprogramming into resolution type macrophages, a vital step required for resolution of inflammation. In day 3 macrophages from VitC sufficient mice,

expression of Gal-1 and 15-Lox mRNA was robust (Figure 21). In contradistinction, enhanced Gal-1 and 15-LoxmRNA expression was delayed to day 5 in VitC deficient macrophages. The delays in resolution we observed in VitC deficient mice were confirmed by quantification of resolvins in peritoneal exudates; increases of which were present only on day 5 (Figure 21). Further confirmation of altered spatiotemporal relationships was achieved by studying macrophage phenotypic changes by examining the distribution of CD11b on macrophages from VitC sufficient or deficient mice on day 3 and day 5 following TG-induced peritonitis (Figure 22). Phenotypic changes in macrophages were accompanied by alterations in macrophage function as demonstrated by the increased susceptibility of VitC deficient macrophages to mitochondrial dysfunction when exposed to reactive oxygen species (Figure 23). In final studies, we employed the human monocyte/macrophage cell line THP-1, which lacks VitC in culture medium when cultured under standard conditions. We demonstrated increased pro-inflammatory gene expression in THP-1 when exposed to LPS under VitC-deprived conditions. Loading THP-1 cells with AscA significantly attenuated mRNA expression of pro-inflammatory genes via a mechanism likely involving reduced activation of the transcription factor NFκB. VitC loading was effective both *in vitro* and *in vivo* since daily AscA infusion following induction of peritonitis significantly restored macrophage phenotype and function in the VitC deficient mice. Few studies have examined the role of VitC in resolution of sterile inflammation. Ganguly et al. initially reported that VitC deficiency affected migration of guinea pig macrophages under *in vitro* conditions.(129) They further showed that addition of exogenous VitC partially restored the migratory response. May et al. showed that activated macrophages use

ascorbate to lessen self-generated oxidant stress.(105) They later showed that ascorbate deficient peritoneal macrophages were more susceptible to H_2O_2-induced mitochondrial dysfunction and apoptosis.(130) However, no studies to date have examined macrophage function during resolution of inflammation in mice lacking the ability to synthesize their own VitC. Our observation of persistence of PMN at the site of inflammation in VitC deficient mice is in agreement with our previous results and those of Vissers and Wilkie who used a similar TG model of peritonitis to show impairment in PMN apoptosis and clearance.(54, 112) It has been suggested that the engulfment of apoptotic cells is generally anti-inflammatory or immunologically silent due to the fact that it sequesters dying cells thus preventing release of potentially toxic cell contents into the local environment.(131) Based on the observations that PMN persists for up to 5 days in the peritoneum of VitC deficient mice (Table 2), it is therefore possible that the apoptosis-resistant PMN can cause strong pro-inflammatory responses from the macrophages that extravasate to sites of inflammation. Indeed strong and persistent pro-inflammatory responses were evident in VitC deficient macrophages elicited on day 3 and even day 5 (Figure 19). Efferocytosis, a process by which dead and/or dying cells are being engulfed and removed by other cells, has been reported to induce production of anti-inflammatory mediators from macrophages that suppress inflammation thereby silently clearing apoptotic cells and thus dampening pro-inflammatory responses.(132) VitC sufficient mice exhibited anti-inflammatory mediator expression in macrophages early (day 3) in the post TG-induced inflammatory process, a phenomenon indicative of functional efferocytosis. In contrast, VitC deficient macrophages failed to upregulate anti-inflammatory mediator production until day 5 (Figure 19). Gal-1 and 12/15-Lox play

vital roles in resolution of inflammation. Rostoker et al. have shown that Gal-1 promotes the generation of M2-like macrophages, which then favors tissue repair during early resolution of inflammation.(127) Ariel and Timor demonstrated that Gal-1 promotes generation of Mres from M2 macrophages, which generates pro-resolving lipid mediators. This phenotype change promotes macrophage departure from peritoneal cavities with resolving inflammation, thus allowing return of tissue to homeostasis.(133) Moreover, Gal-1 expression, which is enhanced inCD11bhigh macrophages, declines sharply as cells revert to the CD11blow phenotype. CD11blow macrophage phenotypes, as noted previously, promote departure from peritoneal cavities with resolving inflammation.(127) Our findings (Figures 21 and 22) which agree with the above studies implicate VitC as a critical regulator of macrophage transition during resolution of inflammation. Expression and function of 12/15-Lox produce key mediators (e.g., lipoxins, resolvins, protectins, and maresins) that promote resolution of pro-inflammatory pathologies.(134) In particular, human and murine monocytes/macrophages expression of 15-Lox is upregulated by efferocytosis with production of mediators such as RvD1, a mediator shown to promote the resolution of murine peritonitis.(125, 135) Further, Gal-1 directly promotes 15-lipoxygenase expression and activity in macrophages during the inflammatory and resolving phases of peritonitis.(127) The earlier increases in Gal-1 and 15-Lox mRNA expression in VitC sufficient macrophages (Figures 21(b) and 21(c)) and the delayed resolvin production in the VitC deficient macrophages (Figure 21(d)) indicate for the first time that VitC influences multiple processes leading to the resolution of inflammation.

3.5 Conclusion

The findings in this mouse model have significant human relevance since VitC levels are subnormal in multiple human inflammatory disease states including sepsis, systemic inflammatory response syndrome (SIRS), trauma, and cancer, among others. In a recently completed Phase I trial (ClinicalTrials.gov identifier NCT01434121) of intravenous AscA in critically ill patients with severe sepsis, we showed that septic patients exhibited abnormally low VitC plasma levels and that intravenous AscA infusion could significantly increase circulating VitC levels.(32) Further, AscA infusion significantly reduced the pro-inflammatory biomarkers C-reactive protein and procalcitonin as well as multiple organ dysfunction.(32) Our findings here add a previously unrecognized element to our understanding of the machinery that governs the resolution of inflammation.

Chapter 4: VITAMIN C IN WOUND HEALING: A NEW PERSPECTIVE

4.1 Intoduction:

Wound healing is a normal physiologic process aimed at restoring the anatomical structure and function of injured skin.(136) Many factors have been identified that affect wound healing like age, wound location, wound size, nutritional status, immune system status, and underlying co-morbidities such as diabetes and obesity.(137, 138) Delayed wound healing can result in a number of complications such as increased hospital length of stay, amputations, and even death.(138) There is a 2 % prevalence of chronic, non-healing wounds in the general population that is associated with an annual estimated cost of > $50 billion and this expenditure is expected to rise in the coming years.(139) Hence there is an unmet need to better identify the mechanisms that delay normal wound healing.

The ultimate goal of wound repair is achieving an adequate restoration of epidermal barrier (wound closure) without a loss of function within a reasonable time frame. Re-establishing the integrity of injured skin necessitates a delicate balance between four sequential yet overlapping stages: hemostasis, inflammation, proliferation and remodeling (maturation).(140) This process involves a highly ordered series of cellular events: platelet activation and fibrin clot formation at the wound site (hemostasis); polymorphonuclear neutrophil (PMN) infiltration to contain invading micro-organisms and clear damaged matrix and tissue debris, followed by macrophage infiltration to engulf and clear apoptotic sated PMNs (inflammation); and fibroblast migration and proliferation (proliferation) to lay down the new matrix collagen that

progressively matures, cross-links, and organizes (remodeling/maturation).(140) These cellular events are orchestrated by chemokines and cytokines such as platelet derived growth factor (PDGF), transforming growth factor beta (TGF-α), vascular endothelial growth factor (VEGF), connective tissue growth factor (CTGF), interlukin-6 (IL-6), interleukin-1β (IL-1β), and tumor necrosis factor alpha (TNF-α)(141, 142) whose expression ultimately determine the course and fate of wound healing.

Wounds create an environment of higher catabolic state.(143) Following injury, the rate at which micronutrients are metabolized increases significantly often leading to critical deficiencies.(143) Indeed, levels of vitamin C (VitC), a small, organic, water soluble micronutrient, with strong anti-oxidant properties, (2, 144) fall rapidly during inflammation. Moreover, scorbutic individuals experience delayed healing and decreased rates of collagen synthesis and maturation.(145) Along with its strong anti-oxidant properties, VitC is an essential co-factor for multiple enzymatic reactions and has recently been shown to suppress pro-inflammatory processes by pleiotropic mechanisms while promoting anti-inflammatory and pro-resolution effects in macrophages.(29, 31, 112, 146) VitC is also intimately involved in collagen metabolism and regulation and therefore many studies have focused on this particular role in wound healing.(147-150) Humans lack functional *L-gulono-□-lactone oxidase* (Gulo), the final enzyme for VitC biosynthesis, and hence are dependent upon an external supply (in diet) of VitC.(2, 144) In contrast, wild type mice express functional Gulo and maintain high levels of VitC in their tissues. In order to better understand the role of VitC in wound healing we used humanized knock-out mice lacking Gulo ($Gulo^{-/-}$) in our studies. In these studies, we went beyond the known effects of VitC on collagen synthesis to

explore the role of VitC on the spatiotemporal changes in the inflammatory, proliferative and maturation stages of wound healing.

4.2 Methods

4.2.1 Animals: All animal studies were performed in accordance to the Virginia Commonwealth University Animal Care and Use Committee's approved protocols. Gulo$^{-/-}$ mice were bred in-house from an established homozygous colony maintained on a C57BL/6J background as previously described.(31) Mice were fed *ad libitum* with regular chow and had free access to water supplemented with AscA (330 mg/L) renewed twice a week to yield *sufficient* mice. Supplements were given in de-ionized water with 20 µL of 0.5 M EDTA/L to increase the stability of the AscA in solution. At week 10 of age, some mice were rendered VitC *deficient* by reducing VitC supplementation for one week (33 mg/L), followed by complete removal of VitC supplementation for an additional week. This reduced supplementation was shown to result in very low plasma VitC concentration, yet insufficient to result in scurvy.(58, 59) In this study, mice were divided into three groups: *VitC sufficient*, *VitC deficient* and *VitC deficient+AscA* mice. The third group was given daily parenteral AscA (200 mg/kg intraperitoneal injection) and AscA supplemented drinking water (330 mg/L) for up to 14 days following wounding.

4.2.2 Surgical Procedure: Animals were maintained under isoflurane anesthesia. In addition, they were also injected subcutaneously with the analgesic buprenorphine (4µg/ml) for pain management. Wounds were created using the methods described by Galiano *et al*.(151) Briefly, the mouse hair on the dorsum of the mice thoracic curvature was shaved with an electric clipper followed by an additional treatment with a depilatory

cream (Nair), for 3 minutes, to remove any remaining fur. The surgical area was then neutralized with betadine followed by another disinfection step with alcohol swaps. Two full thickness excisional wounds were created on the back of VitC *sufficient/deficient* Gulo$^{-/-}$ mice using a sterile 6-mm biopsy punch. Wounds were covered with non-adherent dressing (Telfa™, Covidien); and animals were housed individually with *ad libitum* water and food access as discussed earlier. At day 7 and day 14 post-wounding, mice were anesthetized, blood was collected via cardiac puncture (anti-coagulated with sodium citrate 1:10) and kept on ice to be processed for VitC analysis. Mice were then sacrificed and the wound tissue was collected using forceps and scissors. Per mouse, the tissue from one wound was fixed in formalin for 48hr and processed for histology. The tissue from the second wound was excised, bisected, and stored in liquid nitrogen followed by long term storage at -80^{0}C for subsequent RNA and protein extraction.

Human Neonatal Dermal Fibroblast (HnDF) Culture: Primary HnDF cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in high glucose, Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100µg/mL), and streptomycin (100µg/mL) under a 5% CO_2 atmosphere at 37°C. All experiments were performed using cells at passages 3 to 5.

4.2.3 VitC Analysis: Blood samples obtained from mice on day 7 and 14 post wounding were kept on ice, centrifuged and the resultant plasma was deproteinized as previously described.(146) Briefly, 100µL of plasma was deproteinized with 200µL of cold 20% trichloroacetic acid (TCA), and treated with 200µL of cold 0.2% dithiothreitol (DTT) to prevent oxidation. The mixture was vortexed intermittently for 2 min and

centrifuged (10,000g, 4 ^{0}C, 10 min). Supernatants were stored at -80 ^{0}C for batch analysis using a fluorescence end-point assay.(130)

4.2.4 Histological Staining and Wound Assessment: Formalin fixed paraffin embedded wound sections (3-4 μm) were cleared in xylene washes, rehydrated in a series of decreasing alcohol concentrations and brought to water. Prepared specimens were stained with Masson's Trichrome according to manufacturer's protocol (Richard Allan Scientific, Catalogue # KTRA87019, Kalamazoo, MI).

4.2.5 RNA Isolation and Real-Time Quantitative PCR (QPCR) analysis: Total RNA isolation and real-time QPCR were performed as described previously.(146) Assays were run in triplicate with no template controls and no reverse transcriptase controls. The mRNA expression from a "*sufficient"* mice or a media well was set to "1.0" and mRNA expression of all other samples was compared relative to this sample and represented as a fold change. To normalize for differences in the amount of total RNA added to each cDNA reaction and possible variation in the reverse transcriptase efficiency among the different cDNA reactions, the housekeeping gene 18S rRNA was used. Automated gene expression analysis was performed using the Comparative Quantitation module of MxPro QPCR Software (Agilent). Both the forward and reverse primers for each target are listed in table (1) below.

Table 1. Murine and Human Primers used for qPCR

Name	Sequence 5' to 3'
Murine IL-1β forward	CTGAACTCAACTGTGAAATGCC
Murine IL-1β reverse	CAGGTCAAAGGTTTGGAAGC
Murine TNFα forward	GATGAGAAGTTCCCAAATGGC
Murine TNFα reverse	TTGGTGGTTTGCTACGACG
Murine KC forward	CAATGAGCTGCGCTGTCAGTGCCTGCAG
Murine KC reverse	CTGAACCAAGGGAGCTTCAGGGTC

Table 1. Murine and Human Primers used for qPCR

Murine Mpo forward	CTGGATCATGACATCACCTTGACTCC
Murine Mpo reverse	GATCTGGTTGCGAATGGTGATGTTGTTCC
Murine HO-1 forward	GGTACACATCCAAGCCGAGAATGCTGAG
Murine HO-1 reverse	CGGTGCAGCTCCTCAGGGAAGTAGAG
Murine VEGF forward	GAGACCCTGGTGGACATC
Murine VEGF reverse	CTTTCTTTGGTCTGCATTCAC
Murine CTGF forward	CCCAACTATGATGCGAGCC
Murine CTGF reverse	ACAGGCTTGGCGATTTTAGG
Murine TGF-β forward	TGACGTCACTGGAGTTGTACGG
Murine TGF-β reverse	CCACGTGGAGTTTGTTATCTTTGC
Murine Gal1 forward	CAGCAACCTGAATCTCAAACC
Murine Gal1 reverse	AGTGTAGGCACAGGTTGTTGC
Human IL-6 forward	GGATTCAATGAGGAGACTTGCC
Human IL-6 reverse	TCTGCAGGAACTGGATCAGG
Human Nanog forward	AATGTCTTCTGCTGAGATGCC
Human Nanog reverse	GCTGTCCTGAATAAGCAGATCC
Human OCT4 forward	CTTGCTGCAGAAGTGGG
Human OCT4 reverse	CACTCGGTTCTCGATACTGG
Human p21 forward	CTGTCTTGTACCCTTGTGCC
Human p21 reverse	CCTCTTGGAGAAGATCAGCC
Human p27 forward	TGGACCCAAAGACTGATCC
Human p27 reverse	CATTTTCTTCTGTTCTGTTGGC

4.2.6 Western Blot Analysis: Wound tissues whole cell extract and HnDF whole-cell and nuclear extracts were isolated and used for Western Blot analysis as described previously.(29) Nuclear extracts were isolated using the NE-PER kit (Pierce Biotechnology, Rockford, IL, USA). Antibodies to hemoxygenase-1 (HO-1) (ADI-SPA-896-D, Enzo life sciences, Farmingdale, NY, USA), Oct-3/4 (sc-5279, Santa Cruz Biotechnology, Santa Cruz, CA), lamin B (sc-6216, Santa Cruz Biotechnology), and actin (sc-1616, Santa Cruz Biotechnology, Santa Cruz, CA) were used in this study. Optical densities of antibody-specific bands were determined using Quantity One acquisition and analysis software (BioRad, Hercules, CA).

VitC Uptake by HnDF: HnDF were grown under normal culture conditions to confluence in 12-well plates. Cells were then exposed to media alone or AscA (0.5mM and 1mM) for 3 hours. Media was removed and cells were washed twice with PBS. Fifty µl of tissue culture grade water was added to each well. Cells were lysed by repeated freeze-thaw cycles (x3). Lysates were treated with TCA and DTT and intracellular VitC content was determined as described above.

4.2.7 HnDF Proliferation Assay: HnDF were seeded into 96-well plates at an initial cell density of 3K cells per well. Cells were allowed to rest for a day and then exposed to media only, media + 0.5mM AscA, or media + 1mM AscA for 24 hours. Media was then aspirated and proliferation determined using the CyQUANT® cell proliferation assay kit (Invitrogen, Carlsbad, CA, USA) as per manufacturer instructions.

4.2.8 Healed skin tensile testing: For these set of experiments, mice were sacrificed at day 14 post-wounding and 20mm X 4mm skin specimens were harvested along the mouse central axis. Specimens were wrapped in PBS-moistened gauze and stored at 4^0C overnight prior to testing. Dimensions of each specimen were taken 3 times using a digital caliper and the average was used to calculate the cross-sectional area. Two samples were harvested per animal with the healed wound area located mid-substance. Mechanical testing was performed at room temperature using MTS tensile testing machine and Testworks 4.06A software (MTS Systems, Eden Prairie, MN). The starting conditions were a preload of 0.01 Newtons (N), and tensile grip moving rate of 10 mm/min. Only samples that failed mid-substance were included in the analysis, otherwise samples were excluded (due to failure at the clamp or samples slipped from the clamp).

4.2.9 Statistical Analysis: Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). Data are expressed as mean ± SE. Results were compared using one-way ANOVA. Post hoc Tukey test was used to carry out multi-comparisons between groups. The level of significance for all statistical tests was $p < 0.05$.

4.3 Results

4.3.1 VitC levels affect the progression of wound healing: To examine the effect of VitC on wound progression, three groups of Gulo$^{-/-}$ were generated and subject to the wounding procedure described in the Methods section. We then measured plasma VitC levels on day 7 and 14 post-wounding. As seen in Figure 25A, at day 7 post-wounding, VitC "*sufficient*" mice had significantly higher circulating plasma VitC when compared to the other groups. The VitC levels in the "*deficient +AscA*" group were also significantly higher than the *deficient* group by day 7. By day 14 (Figure 25B), circulating plasma VitC levels in the "*sufficient*" and "*deficient + AscA*" became comparable and significantly higher than the "*deficient*" group. Masson's trichrome stained wound sections on day 7 demonstrated dense granulation tissue and higher collagen deposition (blue color) from "*sufficient*" and "*deficient + AscA*" mice when compared to the wound sections from "deficient" mice (Figure 26 A-C). In both the VitC groups, the dermis and epidermis were better connected to one another. Moreover, the basal lamina and keratin layer were also well developed and presented as distinct layers. In contrast, day 7 sections from "*deficient*" mice demonstrate loose granulation tissue with reduced collagen deposition. Additionally, in deficient mice, the dermis was more

cellular (and reticular) and the lamina was not yet fully distinct (Figure 26 B). Most of these striking differences were also evident on day 14 post-wounding (Figure 26 D-E).

4.3.2 VitC attenuates mediators of inflammation in wound healing: Next we examined the mRNA expression of the pro-inflammatory genes (IL-1 β, KC, TNF-α, and MPO) in healing tissue 7 and 14 days post-wounding. VitC "*sufficiency*" or treatment of "*deficient*" mice with AscA significantly attenuated transcript levels of these pro-inflammatory genes when compared to wound tissue from VitC "*deficient*" mice at day 7 (Figure 27). The transcript levels of the pro-inflammatory signaling cytokines (IL-1β and KC) remained elevated on day 14 in the "*deficient*" mice compared to the "*sufficient*" and "*deficient + AscA*" mice (Figure 28). MPO transcripts however were not detectable in all 3 groups on day 14 (data not shown). TNF-α transcript expression on day 14 showed the same trend as IL-1β and KC but these differences in expression did not achieve statistical significance. In light of the elevated and persistent expression of pro-inflammatory mediator transcripts in VitC deficient animals, we examined whether markers of inflammation resolution might be altered. Galectin-1 (Gal-1) expression is associated with generation of pro-resolving lipid mediators and successful resolution of inflammation.(152) Therefore, we examined Gal-1 expression in day 7 wounds. Gal1 transcript levels were significantly higher in VitC "*sufficient*" mice compared with the "*deficient*" mice (Figure 29). Although Gal1 gene expression trended higher in the "*deficient + AscA*" group, it did not reach statistical significance.

4.3.3 VitC induces genes that promote wound healing: We also examined the expression of transcripts levels of several other known modulators of wound healing including hemoxygenase-1(HO-1), vascular endothelial growth factor (VEGF),

connective tissue growth factor (CTGF), and transforming growth factor beta (TGF-β). On day 7 post-wounding, both VitC "*sufficient*" and "*deficient + AscA*" Gulo$^{-/-}$ mice showed significantly higher mRNA expression of HO-1, VEGF, and CTGF (Figure 30 A-C) in comparison to the "*deficient*" mice. TGF-β expression was significantly elevated in "*sufficient*" mice compared to "*deficient*" mice. TGF-β expression following AscA infusion trended higher in the "*deficient + AscA*" group but did not reach statistical significance (Figure 30D). Induction at the protein level was confirmed by Western blot analysis for total HO-1 (Figure 31).

4.3.4 VitC and tensile strength of healed wound: Using the excisional wound model, samples collected at day 14 post-wounding were used to carry out skin tension studies as described in Methods. Stress at the healing section was measured using the mid-specimen cross sectional area (in KiloPascals). The healed skin samples from the "*sufficient"* mice demonstrated a significantly higher stiffness compared to the other groups (Figure 32). However, there were no statistically significant differences between the "*deficient"* and "*deficient + AscA"* groups. In addition, we also measured the Peak load, a measure of the tensile strength a material can withstand before breaking. However, no significant differences were observed between the different mice groups (data not shown).

4.3.5 VitC promotes HnDF proliferation: Confluent HnDF cells were incubated for 3 hours with two concentrations of AscA (0.5 mM and 1mM). In the absence of exogenous AscA, intracellular VitC concentrations in HnDF were extremely low. There was a dose and time dependent increase in intracellular VitC concentrations following AscA loading (Figure 33) suggesting that these cells have the ability to transport VitC

intracellularly. Additionally, incubating sub-confluent HnDF cells with the same AscA concentrations (0.5 mM and 1mM) for 24 hours resulted in significant dose dependent increases in HnDF proliferation (24% and 40%, respectively) as seen in Figure 34.

VitC induces the expression of self-renewal, cell-cycle progression and fibroblast motility genes in HnDF: To further investigate the increased proliferative capacity brought by AscA, HnDF cells were incubated with AscA (0.5 mM and 1mM) for 3 hours and mRNA expression of the self-renewal genes Nanog and octamer-binding factor 4 (OCT4) determined by QPCR. Both Nanog and OCT4 gene expression increased in a dose dependent fashion following HnDF incubation with AscA (Figure 35). The increased expression was not statistically significant with low level AscA (0.5 mM) but was significant with the higher AscA concentration ($p<0.05$). The mRNA expression levels of the cyclin-dependent kinase inhibitors and regulators of cell cycle, p21 and p27 were also determined (Figure 35). Transcript levels of p27 were significantly decreased in a dose dependent fashion by AscA exposure. However p21 expression levels were unchanged. Western Blot analysis confirmed the increased nuclear expression of OCT4 seen with QPCR at 24 hours post incubation with AscA (Figure 36). The pleiotropic cytokine IL-6 has been shown to favorably promote cell motility and matrix remodeling during the inflammatory phase of wound healing.(153, 154) In addition, IL-6 is a resolution promoting cytokine by virtue of its inhibitory effects on pro-inflammatory signaling via activation of IL-10 and IL-13, and by its ability to enhance polarization of macrophages at wound healing sites towards an anti-inflammatory/alternatively activated phenotype.(155) Therefore we examined IL-6 mRNA expression following exposure of HnDF cells to AscA. As seen in Figure 9F, exposure of HnDF to AscA

produced a dose dependent increase in IL-6 mRNA expression levels; 2.5 fold and 4 fold increase with 0.5mM and 1mM AscA, respectively.

4.4 Discussion

Wounds and more generally injuries are associated with rapid micronutrient deficiencies.(143) Levels of one critical micronutrient, VitC, have been shown to drop significantly (60-70%) at the wound site and not recover completely even after 14 days post-wounding.(156, 157) These observations may reflect VitC depletion by the plethora of free oxidant radicals generated in the wound microenvironment, and in part by the increased consumption of VitC in different biological processes (e.g. collagen synthesis) that are activated during the repair process. While higher oral intake is achievable, attaining significant plasma levels are limited by gastric intolerance and also by limited absorption and renal excretion thresholds.(19) We and others have shown that parenteral VitC is advantageous in bypassing these limitations to produce and sustain adequate plasma levels.(19, 158)

In our present study, we were able to show improved wound healing characteristics with VitC sufficiency and also with i.p. VitC repletion of Gulo$^{(-/-)}$ mice. Day 7 wounds from VitC "*deficient"* mice showed a spatiotemporal gene expression characterized by persistent inflammation combined with delayed resolution and proliferation. The pro-inflammatory state persisted throughout day 14 post-wounds in the "*deficient"* mice but not in the "*sufficient*" or "*deficient + AscA*" groups. In contrast, VitC "*sufficient"* and supplemented Gulo$^{(-/-)}$ mice showed attenuated expression of pro-inflammatory cytokines, increased expression of favorable biomarkers of wound healing, and better expression of the pro-resolution markers of wound healing. However, tangible effects on

the skin tensile strength properties at day 14 post-wounding were evident only in the VitC "*sufficient"* mice. These improvements in wound healing in the VitC "*sufficient"* and supplemented mice were corroborated by *in vitro* cultures of HnDF which showed that VitC supplementation led to robust uptake and fibroblast proliferation, induction of self-renewal genes and up-regulation of a pro-resolution cytokine that also plays a role in fibroblast mobility.

Histological examination of day 7 and day 14 Masson's Trichrome stained sections showed that wounds from VitC "*sufficient"* and supplemented mice had better matrix organization with clear distinction of all skin layers and significant extracellular matrix deposition. In contrast, wounds from VitC "*deficient"* mice were characterized by a loose dermis that was highly cellular and accompanied by limited collagen deposition. At a molecular level, wounds from "*deficient"* mice actively expressed the pro-inflammatory cytokines IL-1β and TNF-α. Additionally, wounds from "*deficient"* mice demonstrated significantly higher MPO expression compared to wounds from VitC "*sufficient"* and AscA infused mice. MPO is an enzyme almost exclusively produced by PMNs (5% of total neutrophil protein) and to some extent in monocytes and is often used as a marker of PMN infiltration.(159) High MPO levels have been found to be associated with poor healing wounds.(160) A similar pattern was observed with the gene expression of the PMN chemoattractant factor, KC. Murine KC is analogous to human CXCL-1 (a functional homologue of IL-8). We interpret the presence of these pro-inflammatory markers as ongoing/sustained pro-inflammatory cellular infiltration into the wound site and an extended pro-inflammatory phase in wounds from "*deficient'* mice. In contrast, the attenuated expression of these pro-inflammatory mediators in VitC "*sufficient"* and

AscA infused mice is indicative of termination of pro-inflammatory cell infiltration to the wound site and perhaps the end of the pro-inflammatory phase. The expression pattern on day 14 further supports this assumption.

The proliferation stage of wound healing is typically characterized by increased expression of the pro-reparative growth factor cytokines such as TGF-β, CTGF, and VEGF.(141) TGF- β plays an important role at all stages of wound healing. In the initial stages, it serves as a chemotactic factor for pro-inflammatory cells as well as fibroblasts. In later stages, TGF- β provides a strong mitogenic signal for fibroblasts. Braiman-Wiksman *et al* have shown that high levels of TGF- β are most beneficial after epidermal closure is complete.(161) In our present work, TGF- β expression levels were significantly higher in day 7 wounds from "*sufficient"* mice. Wounds from mice receiving daily AscA infusion showed TGF- β levels that trended higher compared to "*deficient"* mice but did not reach statistical significance. Another growth factor in the wound milieu is CTGF whose transcription is regulated mainly by TGF- β. It is believed that it is a downstream mediator of some of the actions of TGF- β.(162) However, TGF- β independent regulation of CTGF has also been reported.(163) Recently, Alfaro *et al* (2013) reported that CTGF levels at wound sites increase during the proliferation phase.(164) CTGF functions in promoting granulation tissue formation (fibroblast cell division and migration and fibroblast matrix deposition e.g. collagen type I and fibronectin) and wound remodeling.(165) In our study, VitC "*sufficiency"* and AscA supplementation were associated with significantly higher CTGF expression compared to the "*deficient"* mice at day-7 post-wounding ($p<0.05$). Combined with histological evidence, these results indicate an active cellular and molecular proliferation in the VitC

"*sufficient"* and AscA supplemented mice versus an attenuated/lagging proliferation response in "*deficient"* mice. Several studies have shown that VEGF significantly promotes angiogenesis and neovascularization in healing wounds.(140) Multiple factors such as pH, reduced oxygen tension, increased lactate as well as pro-inflammatory cytokines have been reported to induce VEGF production at the wound site.(140, 142) In our study, we observed higher VEGF expression in the VitC "*sufficient"* and AscA infused mice indicating increased angiogenesis and neovascularization in the wounds of "*sufficient"* and AscA supplemented mice. A role for HO-1 in wound healing has only been recently described.(166) Grochot-Przeczek *et al* showed that HO-1 inhibition adversely affects wound healing in diabetic mice while HO-1 over-expression promoted healing.(166) Earlier, the same research group showed that HO-1 upregulated VEGF production and therefore could indirectly promote neovascularization. In our study, HO-1 expression levels were significantly higher in VitC "*sufficient"* and AscA supplemented mice. In sum, the expression pattern of these growth promoting factors can be interpreted as a robust biological response to wounds in VitC "*sufficient"* mice when compared to the "*deficient"* mice. In addition, daily i.p. VitC supplementation seems to restore the growth promoting response in wounds of VitC "*deficient"* mice.

Physiologic wound healing involves a regulated fibroblast differentiation into myofibroblasts that play an active role in reconstruction of damaged tissue following injury.(167) A critical mediator of this process is Gal-1, a lectin produced by various tissues. Recently, Lin *et al* (2015) showed that Gal-1 induced myofibroblast activation and proliferation. Moreover Gal-$1^{(-/-)}$ knockout mice experienced reduced myofibroblast migration, which was corrected with topical Gal-1 administration in excisional wound

models.(168) Elsewhere, Perzelova *et al* showed that exposure of dermal fibroblasts to Gal-1 stimulated extracellular matrix production that subsequently supported endothelial cell growth.(169) In our studies, only VitC "***sufficient"*** mice had significantly higher Gal-1 expression levels as compared to other groups. We have previously shown that both VitC "*sufficiency*" and VitC supplementation were able to induce early Gal-1 expression in peritoneal macrophages, which correlated with markers of resolution.(146) This suggests that VitC may be required to sustain Gal-1 expression in healing wounds and that the level of supplementation provided to the "*deficient*" mice is probably insufficient to adequately restore Gal-1 expression in healing wounds.

Much to our surprise, only VitC "*sufficient*" mice demonstrated significantly higher healed-skin stiffness compared to the other groups despite the observed increased collagen deposition evident by histological analysis in the "***sufficient"*** and "*deficient + AscA"* mice. Although by day 14, we had achieved plasma VitC levels in the "*deficient + AscA*" group that were comparable to the VitC "*sufficient*" mice, it is possible that the VitC levels in the healing wound may not have reached the same level as the sufficient mice. While humans have evolved over millennia to up regulate the VitC transporter SVCT2 at times of deficiency (170) it is unknown whether SVCT2 is up regulated to the same extent in the healing wounds of these knockout mice. Indeed it has been shown that the knockdown of SVCT2 in bone marrow derived stromal cells decreased wound healing, and that supplementing with VitC failed to rescue these cells.(171) Therefore, we interpret these results as insufficient uptake of VitC in the healing wound and that perhaps, an additional topical application of VitC at the wound site or a higher dose of i.p. VitC could prove beneficial.

Using HnDF cells, we showed that AscA exposure resulted in a dose dependent and significant increase in intracellular AscA uptake and fibroblast proliferation. This was associated with a dose dependent inhibition of p27 expression. The inhibitors p21 and p27 suppress cell cycle progression by inhibiting cyclin-dependent kinases as well as by down regulating expression of other genes involved with cell cycle progression.(172) Conversely, AscA treatment was associated with a dose dependent increase in the expression of pluripotent and self-renewal genes Nanog and OCT4.(173) These findings suggest a crucial role for VitC in regulating fibroblast proliferation. Although this is a novel finding in fibroblasts, there is precedence for these actions of VitC in stem cells, and in particular the adipose derived stem cells wherein VitC has been used to increase the yield and regenerative potential of adipose derived stem cells.(174)

IL-6 is a cytokine with pleotropic functions that is produced by several cell types in the wound microenvironment including fibroblasts.(153, 175) It plays an important role in development of Th17 cells and is known to contribute to the inflammatory component of autoimmune diseases.(176) However, recent studies suggest a pro-resolution and anti-inflammatory role for IL-6 in wound healing environments.(155) Kuhn *et al* showed that inhibition of IL-6 resulted in impaired wound healing due to decreased epithelial proliferation.(177) IL-6 knockout mice exhibit delayed wound healing (delayed re-epithelialization, granulation tissue formation, and sub-optimal inflammatory response) compared to wild type mice; this could be reverted with recombinant IL-6.(178, 179) Further, Gallucci *et al* reported IL-6 signaling to promote dermal fibroblast differentiation into myofibroblasts, increase their motility and also promote matrix remodeling.(153, 180) We found that exposure of HnDF cells to AscA promoted a dose dependent

increase in IL-6 mRNA levels. These findings suggest a novel mechanism by which VitC could promote fibroblast motility and matrix remodeling. Presumably, insufficient fibroblast VitC levels could impair IL-6 expression and delay the resolution of wound healing.

Thus, on the basis of these *in vitro* studies, we speculate that in the absence of adequate VitC supplementation, the proliferation and maturation phases of wound healing would be delayed or would remain incomplete, thereby resulting in non-healing wounds.

4.5 Conclusion:

Wound repair is a complex process that requires the co-ordination of various local cellular and biochemical events. To date, the majority of studies have used oral supplementation to augment plasma/tissue levels of VitC. However, recent studies by Padayatty *et al* and others have shown that oral supplementation does not provide the requisite VitC levels for restoration of plasma VitC levels.(19, 59) We have recently shown that parenteral routes of VitC administration are safe and effective in critical care situations in both mice and humans.(158) Moreover, these doses of VitC, when administered parenterally, were sufficient to restore circulating plasma VitC levels. The current study presents new evidence that beyond its well characterized role in collagen metabolism, vitamin C status plays a crucial role in orchestrating multiple wound healing processes. Vitamin C repletion by parenteral infusion has the potential to be a safe and inexpensive therapy for enhancing tissue repair and shortening healing time.

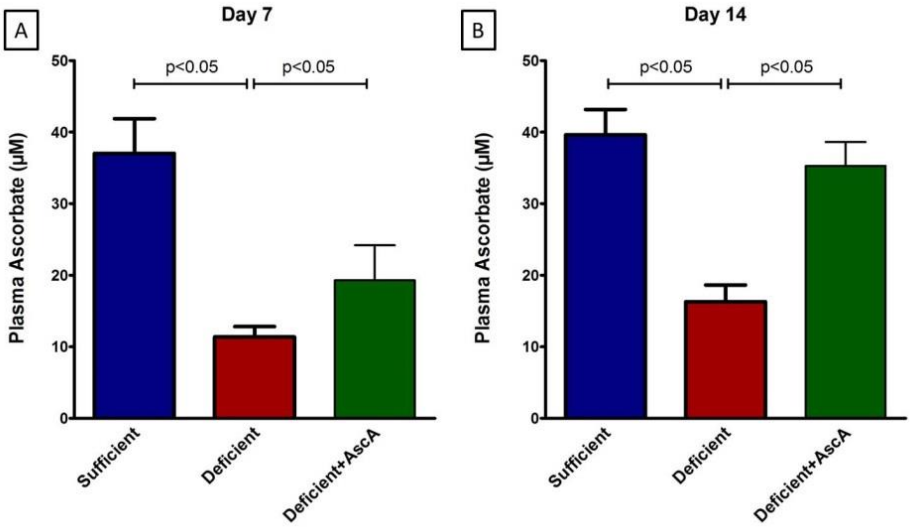

Figure 25: Plasma VitC levels from VitC "sufficient", "deficient" and "deficient + AscA" Gulo$^{-/-}$ mice at day 7 (a) and day 14 (b) post wounding. Daily supplementation of deficient mice with i.p. AscA post-wounding resulted in significantly higher plasma VitC levels in the "deficient + AscA" mice compared to "deficient" mice by day 7 post wounding. These levels further rises and became comparable to sufficient mice plasma VitC levels at day 14 (n= 3–6 mice/group, ns= not significant).

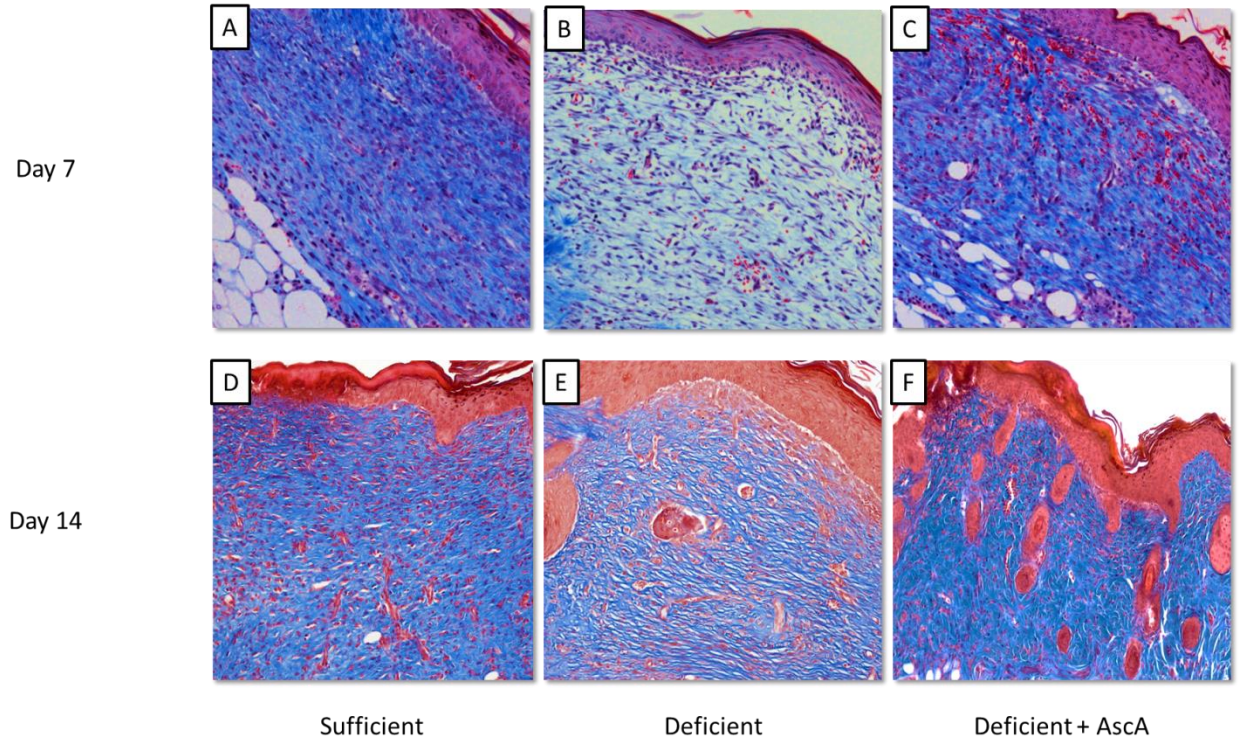

Figure 26: Representative Masson's trichrome stained sections from VitC "sufficient", "deficient", and "deficient + AscA" Gulo$^{-/-}$ mice at day 7 (A-C) and day 14 (D-F) post wounding. VitC "sufficient" (A) and "deficient + AscA" (C) sections were showing more dense granulation tissue with significant collagen

disposition and distinct lamina. In contrast, sections from the "deficient" mice demonstrated a more cellular loose granulation tissue with little collagen disposition (B). Also the lamina was not yet distinct. Day 14 sections were more mature. However, sections from the VitC "deficient" mice were still showing a less compact collagen disposition.

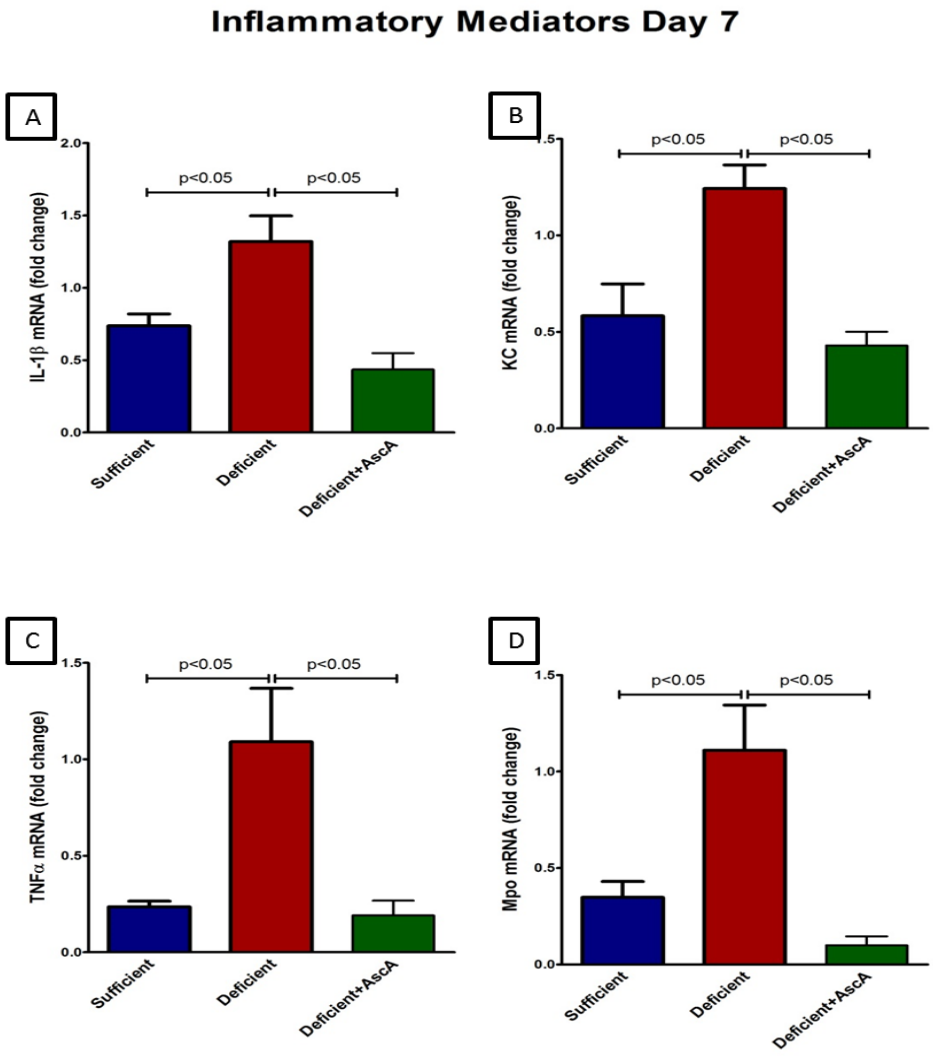

Figure 27: Real time QPCR for IL-1β, KC, TNFα, and Mpo mRNA from day 7 wounds of VitC "sufficient" "deficient", and "deficient + AscA" Gulo$^{-/-}$ mice. Wounds from Vitamin C "sufficienct" and "deficient + AscA" mice demonstrated an attenuated pro-inflammatory gene expression profile compared to "deficient" mice. (n = 3-6 for each group).

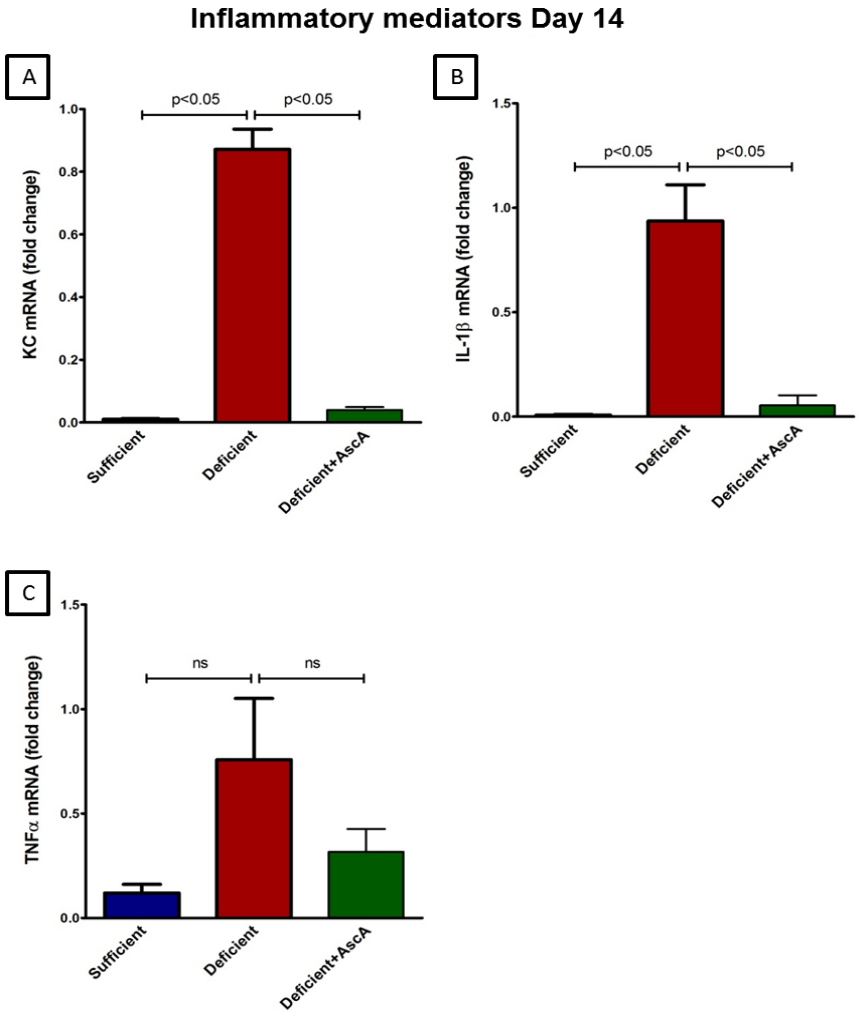

Figure 28: Real time qPCR for IL-1β, KC, and TNF-α mRNA from day 14 wounds of VitC "sufficient" "deficient", and "deficient + AscA" Gulo$^{-/-}$ mice. Wounds from Vitamin C "deficient" was still experience a heightened pro-inflammatory gene expression response compared to VitC sufficient/supplemented mice. (n = 3-6 for each group).

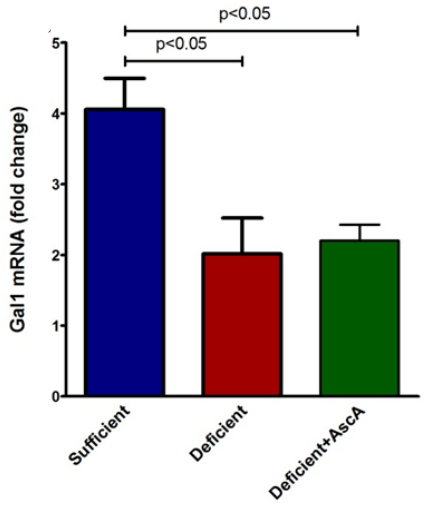

Figure 29: Real time qPCR for Gal1 and Ym1 from day 7 wounds. VitC sufficiency is associated with significantly higher Gal1 expression. VitC "sufficient", but not "deficient + AscA", showed significantly higher Gal1 expression compared to "deficient" wounds at day 7.

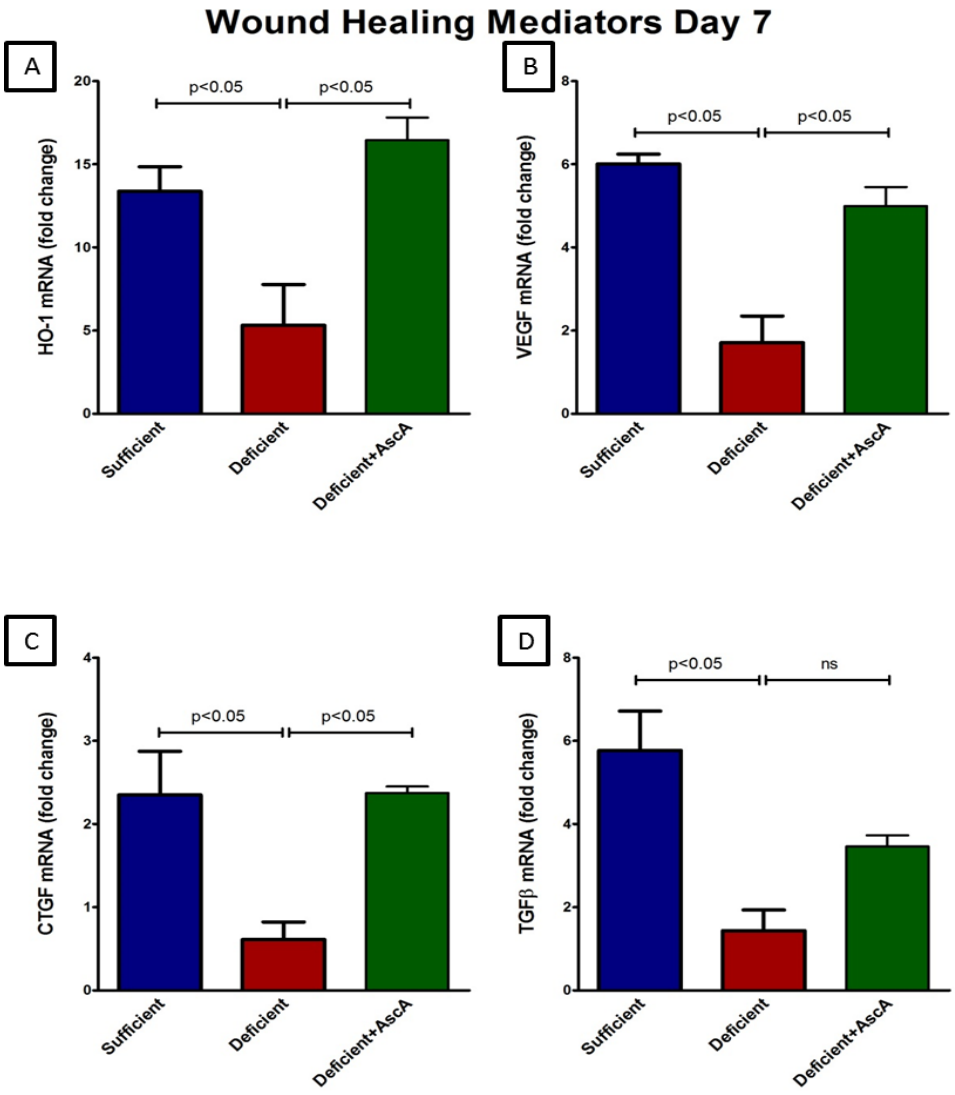

Figure 30: Spatiotemporal profiling of growth factors and HO-1 genes expressions in day 7 wounds. Real time QPCR for (TFG-B, CTGF, and VEGF) , and HO-1 mRNA from day 7 wounds from VitC "sufficient", "deficient", and "deficient + AscA" Gulo$^{-/-}$ mice. (n = 3-6 mice/group, ns = not significant).

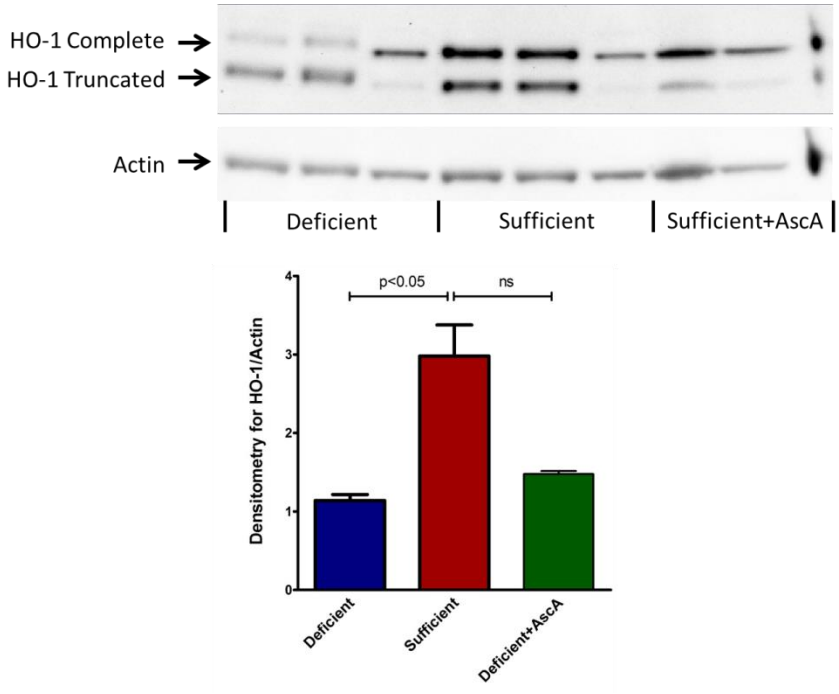

Figure 31: Wound Healing is delayed in Vitamin C deficient wounds. Representative Western blot for expression of HO-1 and actin from day 7 wounds of VitC "sufficient", "deficient", and "deficient + AscA" Gulo$^{-/-}$ mice. The bar chart is a quantitative representation of the western blot results using densitometry (n = 3 - 6 for each group).

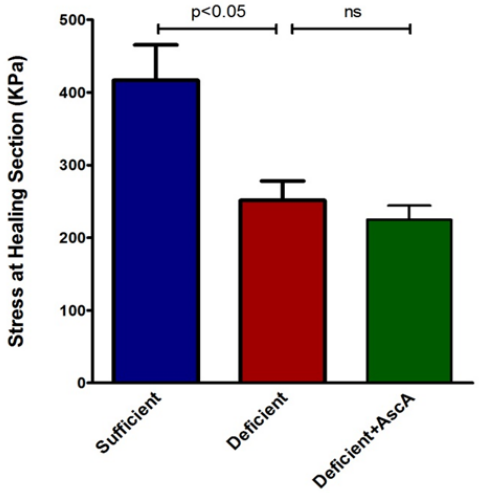

Figure 32: Stiffness in KiloPascal recorded from skin tension studies performed on wounds collected on day 7 post incisional wounding. VitC sufficient mice demonstrated a significantly higher stiffness (calculated at mid specimen) compared to the other groups. There was no statistically significant difference in the wound stiffness between the "deficient" and "deficient + AscA" groups. (n = 6-12; ns = non-significant).

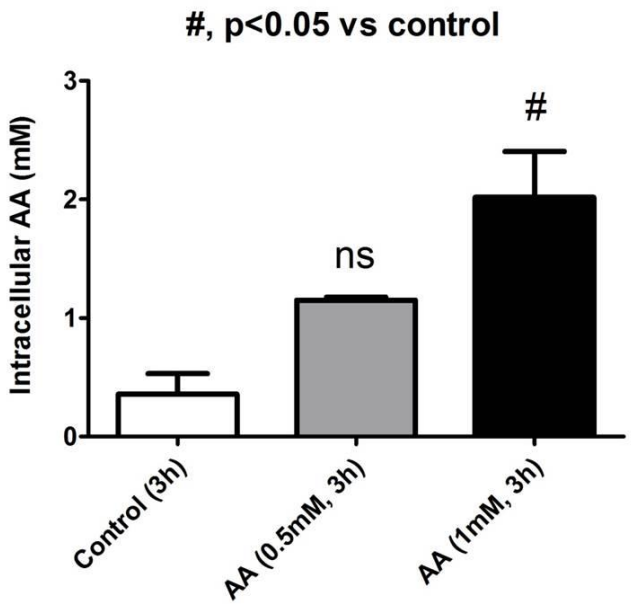

Figure 33: VitC levels measured in HnDF cells after being loaded with AscA under normal culture conditions for 3 hrs at 37°C. AscA concentrations were determined as described in Methods (n = 3 for each group).

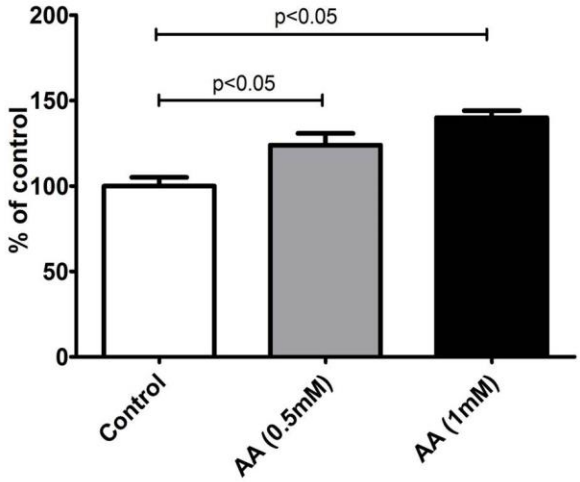

Figure 34: HnDF loading with AscA resulted in dose dependent increase in proliferation (n= 3 for each group).

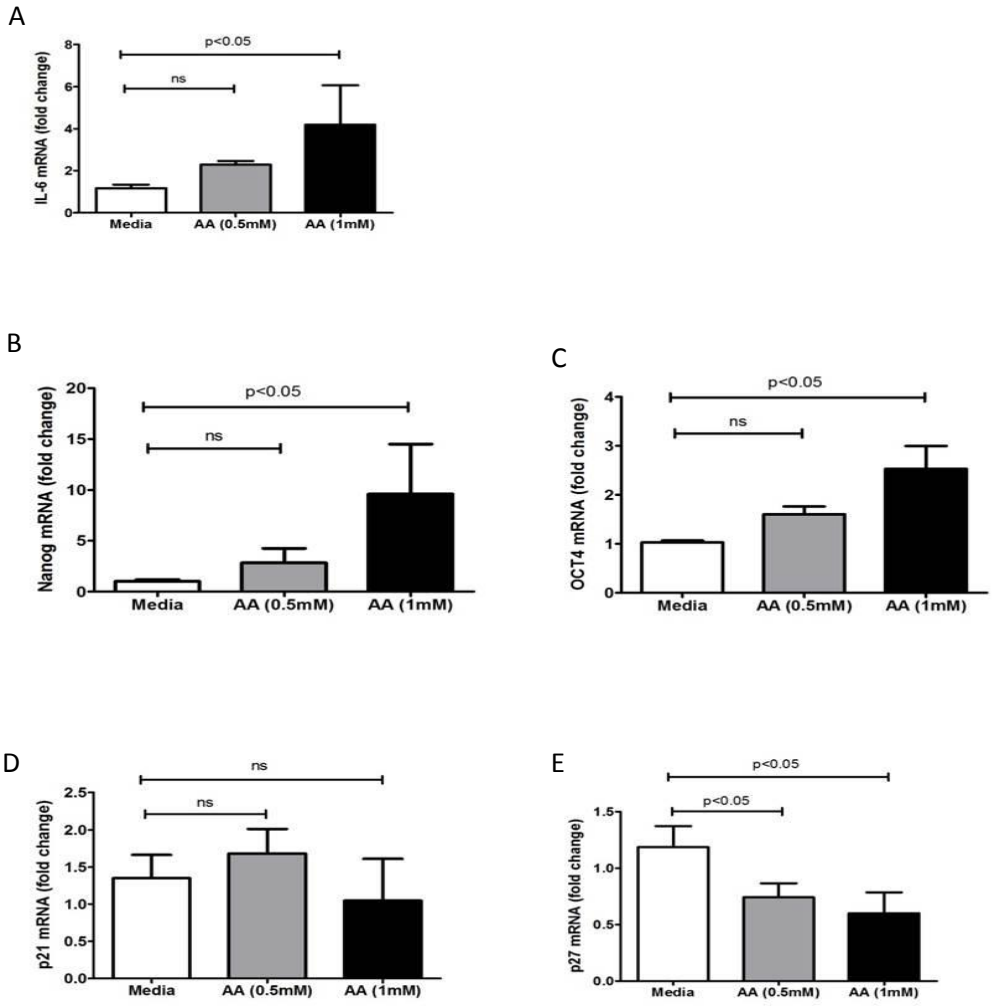

Figure 35: Real-time qPCR for interleukin-6 (IL-6), Nanog, Oct4, p21, and p27 from HnDF cells loaded with AscA 0.5mM and 1mM for 3 hrs (ns: not significant; n = 3 for each group).

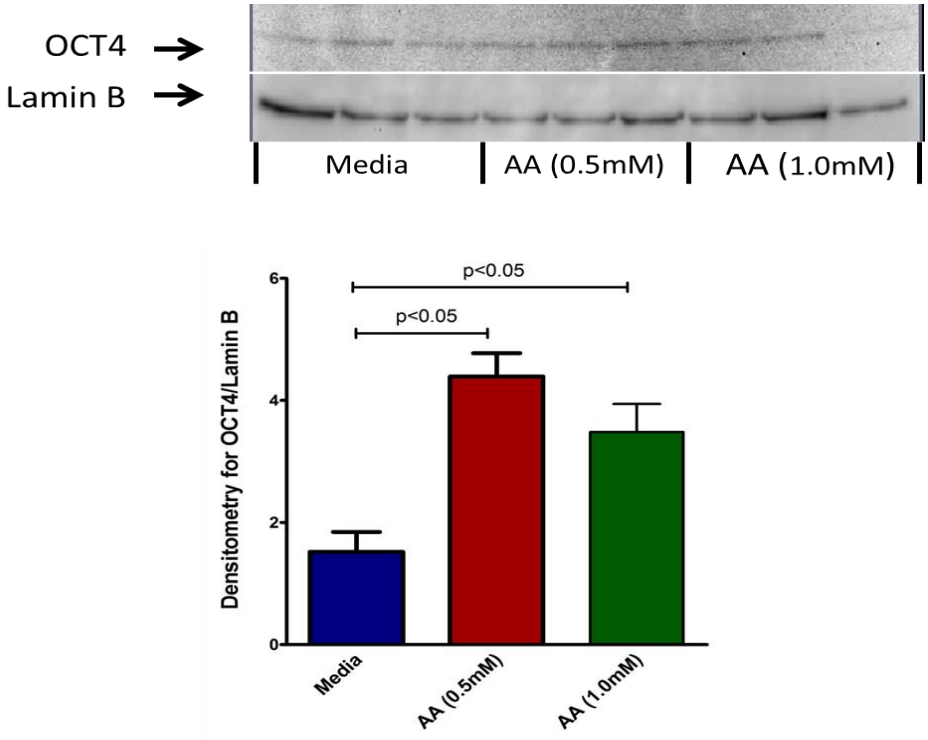

Figure 36: : Representative Western blot for nuclear expression of Oct4 and lamin B from HnDF cells loaded with AscA 0.5mM and 1mM for 3 hrs. The bar chart below is a quantitative representation of the Oct4 western blot results using densitometry (*N* = 3 - 6 for each group).

Chapter 5: Impact of High Dose Ascorbic Acid on Inflammatory markers *and* Atrial Fibrillation among Cardiac Surgery Patients

Future Direction - Study Protocol - Work in Progress

5.1 Abstract

Ischemia-perfusion injury is an inevitable event taking place during open heart surgery involving cardiopulmonary bypass (CPB). Reactive oxidant species (ROS) and the resultant oxidative burst have been demonstrated to be the main pathway for inducing injury during this event. Both the inflammatory and coagulation systems play a role in injury during CPB and are well estab-lished contributors to the oxidative burst. Previous work in this area has shown a significant de-crease in the body's anti-oxidant capacity as the oxidative stress builds. Interestingly, Vitamin C levels, an important dietary anti-oxidant, are reported to decrease after surgery in a similar fash-ion corresponding to the increased oxidant species. Often a decreased systemic anti-oxidant capacity is seen and the body does not revert to normal levels until several days after surgery. The role of Vitamin C has also been emphasized after reports of its use in preventing atrial fibrillation (AF), which is known to occur following CPB surgery with the highest incidence occurring by the third day after surgery. The pathogenesis of AF is suspected to be multifactorial, with ev-idence to support an inflammatory mediated response with a prominent ROS mechanistic com-ponent. The results associated with the use of Vitamin C have been significant when given in-travenously in high doses. On the other hand, studies employing oral administration have demonstrated some controversy in establishing efficacy. Administration of Vitamin C via oral route result in

plasma concentration tightly maintained around 150-250uM. At this level, the Vit-amin concentration may not be enough to replenish tissues and cells and attaining mM concen-tration is needed to compete for the oxidant species. Parenteral administration opens the door for mM concentration to be readily achieved with minor inconveniences. Based on this evidence, the main hypothesis is: “Parenteral administration of high dose Vitamin C will decrease the ROS-mediated inflammatory/coagulopathies among patients undergoing open heart surgery involving CPB”.

5.2 Public Health Impact (Project Narrator)

Cardiac surgery is a leading consumer of health care resources. In 2010, data collected from 1,001 hospitals representing nearly 80% of all sites in the United States that perform coronary artery bypass grafting (CABG), demonstrated that over 150,000 major procedures involved CABG; 18,008 involved both aortic valve replacement and CABG; 2,378 involved mitral valve replacement and CABG; and 4,635 involved mitral valve repair and CABG. The mean hospital charges for CABG and valve procedures were $124,404 and $171,270 with a mean length of hospital stay of 9.1 and 11 days and an in hospital death rate of 1.75% and 3.90%, respectively.(181-183) AF, a known complication of cardiac procedures, is the most common type of arrhythmia experienced following cardiac surgeries with the risk being highest by third post-operative day. AF occurs in up to 40% of the patients and has been associated with poorer prognosis.(26, 184) It ac-counts for increased length of hospital stay, as well as increased morbidity (e.g. stroke) and mortality.(25) It is now supported that the high levels of ROS achieved in the myocardium can lead to both electrical and structure remodeling of the cardiac

muscle leading to the development of AF.(26) Therefore, the administration of safe, inexpensive Vitamin C using the parenteral route is a reasonable novel method to decrease the ROS for patients undergoing CABG and CPB.

5.3 Specific Aims

Cardio-pulmonary bypass (CPB) is considered to be a major contributor of inflammatory injury during cardiac surgery. Recently, the role of reactive oxidant species as a prominent media-tor/stimulant has been defined, recognizing the inevitable oxidative burst which occurs during surgery. Several trials have examined interventions aimed at controlling and attenuating this oxidative burst. Other trials have investigated Vitamin C's ability to halt the oxidant species fueled processes. Unfortunately, the dual routes of administration employed, more oral than in-travenous, have made the results of these studies controversial. With oral dosing, the fasting plasma concentrations are tightly controlled to below 150-200μM.(19) In contrast, with intravenous administration of high doses, significantly higher plasma concentrations in the milliMolar (mM) order were achievable.(19) These facts agree with the principle that the efficacy of an anti-oxidant is dependent upon its concentration in the medium where the oxidant species are generated and the damage is anticipated.(185) The mM concentrations attainable with parenteral administration provide potential for wider therapeutic

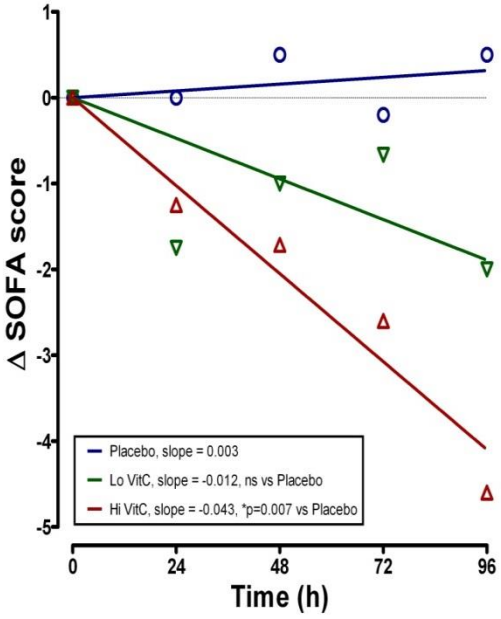

Figure 37: Parenteral Vitamin C (200mg/kg) reduced multiple organ dysfunction (SOFA) score in septic patients.

applications of Vitamin C. A number of trials utilizing comparable and higher doses than proposed in our work have demonstrated improvement in clinical outcomes and decreased overall morbidity and mortality in different patient population having some common pathology. A recent study by our team demonstrated that parenterally administered high dose Vitamin C attenuated sepsis-induced inflammation and coagulopathies in animals, and showed favorable trends in a safety trial of the dose in humans (Figures 1 and 2).(186) Therefore, we hypothesize that administering VitC in

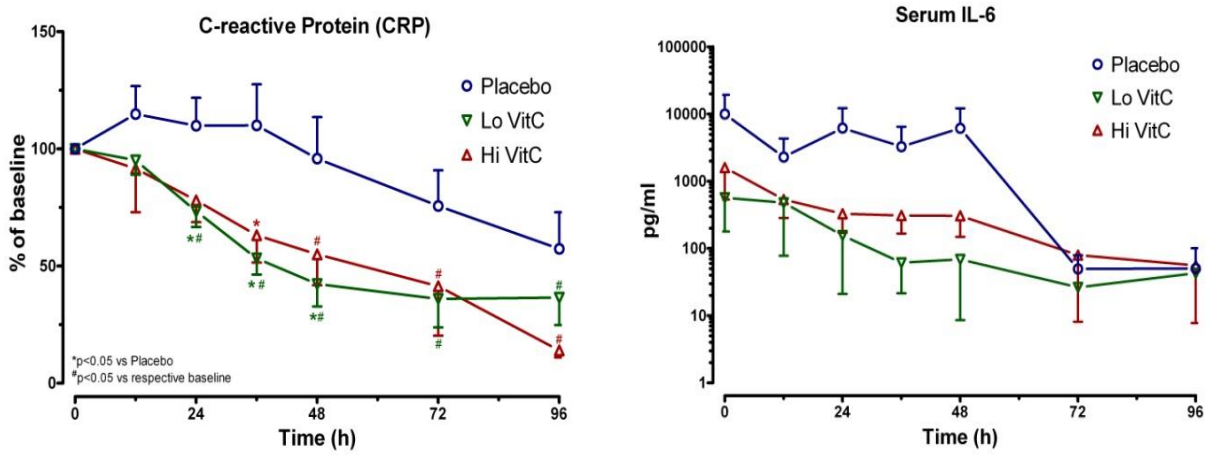

Figure 38: Parenteral Vitamin C (200mg/kg) reduced inflammatory biomarkers in septic patients

a dose of 200mg/kg/day divided into 4 doses per day, as used in our previous work, at the time of cardiac surgery (prior to CPB, during bypass and through post-op day 3) will reduce inflammatory and coagulatory mediators and biomarkers by attenuating the oxidative burst developing during CPB.

5.3.1 Specific Aim 1: Establishing the effects of high dose parenteral Vitamin C on the inflammatory and coagulatory system.

a. Determine how the levels of circulating pro-inflammatory biomarkers profiles are affected.

b. Examine the effects of high dose parenteral Vitamin C on coagulation biomarkers and platelets function.
c. Investigate the effects of high dose parenteral Vitamin C on the cardiac enzymes profiles.
d. Measure vitamin C levels in both groups to draw correlation with blood biomarkers.

It is expected that Vitamin C intervention will result in a significant decrease in the plasma peak levels of inflammatory and coagulatory markers over time as compared to a control group.

5.3.2 Specific Aim 2: Determine the effects of high dose parenteral Vitamin C on patient clinical outcomes: Incidence of AF, ICU length of stay, overall Hospital length of stay, length of time on mechanical ventilation, chest tube volume output and time to wound healing.

Note: This pilot study will not be fully powered to answer specific aim #2 but preliminary data will be collected on outcomes to support larger studies to follow.

5.4 Research Strategy

5.4.1 Background and Significance Cardiac surgery represents a high complexity technical medical intervention. Patients presenting for cardiac surgery have changed over the last 10 years, with clear trends towards treating elderly patients with complex disease states such as congestive heart failure (CHF), worsening secondary diseases, increasing numbers of mechani-cal support device placements and associated complicated medical treatments. Still, the majori-ties of surgeries are coronary bypass

and valve repair cases, which involve CPB. The body and heart are subjected to inflammatory insults through varied causes: surgical trauma, blood contact with the artificial surface of the CPB machine, changing of blood flow type, heparin antico-agulation, micro-embolism, endothelial thrombin release, free iron and hemoglobin generation, blood transfusion and myocardium ischemia-reperfusion injury.(36) Indeed, there is a plethora of literature describing the inflammatory and coagulopathy response induced by CPB.(187) The ischemia-perfusion injury: Following cardioplegia and during CPB, the heart is subjected to is-chemia of varying lengths dependent upon the nature of the operation. The cardiac tissue at that point is electrically resting and its metabolic demands are accordingly low. There is also a severely diminished supply of oxygen and nutrients. Accordingly, ROS generation is low and together with the reduced metabolism result in a slow and direct myocardium cellular damage which extends as the duration of the ischemia increases.(36, 185) However, an actual peak of the oxi-dative stress occurs shortly after reperfusion with oxygenated blood because of the already ac-cumulated oxidative substrates and cellular depletion of reductive counterparts.(185) While the for-mer is the major contributor to cardiac damage related to oxidative burst, it is worth noting that ischemia-reperfusion is not the only contributor to oxidative stress experienced during surgery in this particular patient population, which often has many other co-morbidities. Most significantly of these co-morbidities are diabetes and Coronary Artery Disease (CAD) whose pathologies have a significant ROS injury component. In addition, insults from the operative procedure result in activating the inflammatory and coagulation systems which contribute significantly to oxidative stress through a number of self-perpetuating feedback loops and are further up-regulated

by oxidative stress. McColl A. J. et al. (1998) demonstrated that the total plasma antioxidant status was significantly lower at 1.5hr, 6hr, 24hr, and 72hr after CPB. They further reported that the larger drop was between the 0hr and 1.5hr time points when the elevation in the lipid peroxide level was at the lowest level. The study also evaluated the cardiac troponin T which was elevated at all-time points postoperatively with a significant correlation between the lipid peroxide elevation at 1.5hr and troponin T elevation.(188) In another study, involving 79 patients undergoing CABG involving CPB, showed a significant increase in both total peroxide and oxidative stress index. The stress index continued to be elevated for 48 hours post operatively with a significant decrease in total antioxidant capacity.(189) Furthermore, as reviewed by Larmann, J, & Theilmeier, G. (2004), the endothelium, platelets and leukocytes are activated during CPB.(36) This activation is caused in part by ROS as well as pro-inflammatory mediators, an activated complement system and endotoxins released from the gut which have an ROS component as well.(36) More recent, the role of ROS-mediated inflammatory responses in the development of AF has been established. AF is the most common type of arrhythmia experienced following cardiac surgeries with highest risk on the third day of surgery. It occurs in up to 40% of the patients despite several new therapies.(26) The development of AF post operatively has been associated with poorer prognosis.(26, 184) It accounts for increased length of hospital stay, as well as increased morbidity (e.g. stroke) and mortality.(25) High levels of ROS achieved in the myocardium cause both electrical and structural remodeling of the cardiac muscle leading to AF.(26) An association of more AF with increased transfusions among CPB patients was also reported, thus recognizing the

deleterious effects of the increased plasma load of inflammatory markers and mediators from the transfused red cells.(25)

Interestingly, the concentration of Vitamin C decreases significantly during surgery, sepsis, burns, postoperatively and with postoperative complications.(25) Indeed, Vitamin C levels during and after cardiac surgery are low. Vitamin C serves as a one or two electron-reducing agent ca-pable of seeking out several types of reactive oxidant species.(30, 184) A number of randomized con-trolled trials involving trauma patients, major burns, critically ill cardiac patients as well as patients with subarachnoid hemorrhage employed intravenous (IV) vitamin C supplementation. Doses used were between 1gm every 8hours to 66mg/kg/hr (110gm/24hours for a 70kg patient). A significant improvement in patient outcomes was observed. There was a decrease in patients' ICU length of stay, hospital length of stay, duration on mechanical ventilation, the inflammation marker C-reactive protein (CRP), wound edema, time to wound healing and an overall decrease in morbidity and mortality.(25) Recent research in cardiac surgery has suggested that the use of Vitamin C decreases AF. Although, the incidence of AF in one study was as low as 16.3% compared to 34.9% in the control group(25), the results from other cardiac studies regarding AF demonstrated increased variability. Many of these studies employed Vitamin C orally in a relatively low dose, which is thought to be less effective. Vitamin C plasma concentration is usually maintained and controlled below 150 - 250uM with oral supplementation.(19) Oral doses in excess of 200 mg are associated with no further increases in circulating Vitamin C levels.(19) In contrast, when administered intravenously higher plasma concentrations (many folds higher-milliMolar concentrations) are achievable.(19) This is

significant when considering factors such as hemodilution and the associated decrease in the plasma antioxidant capacity. The parenteral route is then considered most effective, and serves making Vitamin C a “new (emerging) drug” to be used in conjunction with current therapies for patients undergoing CABG. Finally, concerns regarding high doses Vitamin C have been raised before. Fortunately, the safety of this dose has been verified in our preliminary human data and the safety of higher doses has previously been verified in cancer trials.(190) The only known reported toxicity of Vitamin C originates after continuous (days to weeks) of infusions or intake and is limited to oxalate renal calculi.(36) Another potential concern was the hypothesized pro-oxidant property of high dose Vitamin C. However, evidence supporting this claim in literature is weak and the antioxidant property at even higher doses was demonstrated to be predominant.(40) Therefore, overall it is a very safe and low risk of toxicity intervention over a wide range of doses including the suggested dose in this proposed work.

5.4.2 Innovation The proposed work is able to combine critical review of literature and innovation for the following reasons: 1) Using high dose Vitamin C, 200mg/Kg/day, intravenously will allow for the achievement of effective high plasma concentrations, 2) First time to employ the novel intervention among the patient population undergoing cardiac surgery involving CPB. With the first dose administered before the onset of CPB, the body tissues will have the opportunity, most importantly cardiac tissues, to replenish their storage of this natural anti-oxidant as well as in-crease the plasma anti-oxidant capacity, and 3) The interventions as described aim to interfere with the inflammatory and ROS perpetuated mechanisms involved in developing atrial

fibrillation as well as the associated increase in coagulopathies among this patient population during the peri operative period. This pilot study will allow us to detect favorable trends through monitoring of key markers of these interacting systems.

5.4.3 Approach

Specific Aim 1: Establishing the effects of high dose parenteral Vitamin C on the inflammatory and coagulatory system.

a) Determine how the levels of circulating pro-inflammatory biomarkers profiles (CRP, IL6 and TNF-α) are affected. CRP: is a known general marker of inflammatory processes, as well as, an acute phase reactant. It is produced in the liver and is thought to bind phosphotidylcho-line which is in cell membranes. Cell membrane breakdown occurs with ensuing endothelial cell death. It is thought that vitamin C, as an anti-oxidant could/should protect endothelial cells form oxidative stress and early cell death after ischemia and reperfusion injury and hence reduce the level of this biomarker. IL-6, and TNF-α: are interleukins, or cell signaling proteins that white cells release to attract/up regulate other leukocytes. A wide range of inflammatory processes will turn on leukocytes to release these proteins and again it is thought that if vitamin C acts by decreasing the body's response to inflammation form CPB that these biomarkers of inflammation should be lessened.

b) Determine the effects of high dose parenteral Vitamin C on coagulation biomarkers and platelets function. Fibrinogen: is the most important circulating coagulation protein in that it is the building block for solid clot. Fibrinogen is, like CRP, a liver produced acute phase re-actant. Fibrinogen rises as a result of inflammation. Soluble thrombomodulin: is released from intact endothelial cells in response to thrombin

production or active coagulation espe-cially as a way to curb run away pro-coagulant responses. Platelet and WBC counts: change as a response to inflammatory mediators. On the other hand, Rotem is a whole blood clot analysis technology that is particularly sensitive to the interactions of fibrinogen and platelets. As such it is predictive of which patients will bleed after CPB Therefore if vitamin C manages to decrease overall inflammation; we expect Rotem analysis of clot dynamics to demonstrate a better preservation (homeostasis/buffering).

c) Determining the effects of high dose parenteral Vitamin C on cardiac enzyme profile. CPB procedure results in ischemia mimicking myocardial infarction and more profound is the combination of ischemia-reperfusion injury leading to myocardial cell injury, which causes the release of several cardiac tissue markers. Cardiac Troponin I (cTn-1) is considered one of the best predictors of myocardial cellular injury because it is specific to the heart and it lacks cross reactivity with isoforms derived from skeletal muscles. Elevated cTn-I is usually associated with poor clinical outcomes. It is our hypothesis that Vitamin C, by attenuating the ischemia-perfusion injury, may lower peak cTn-I levels and thus indicating lower levels of tissue injury.(191, 192) A limitation of this assay is that the surgery itself and handling the heart could give rise to this marker regardless of the ROS induced injury. However, this marker will be nearly constant among the patients as each patient will experience the same surgery procedure by a limited team.

d) Vitamin C levels will be measured in both groups to draw correlation with the previously de-scribed markers.

Specific Aim 2: Determine the effects of Vitamin C on patient clinical outcomes: incidence of AF, ICU length of stay, overall Hospital length of stay, length of time on mechanical ventilation, chest tube volume output and time to wound healing. The incidence of post-op AF is known to be highest by the third day of surgery. Evidence from the literature supports the inflammatory and ROS mechanistic components of AF development and perpetuation. It is expected that high dose Vitamin C should be able to attenuate considerably the inflammatory and ROS insults. Patients will be followed up through discharge to record the incidence of AF by reviewing clinical notes for any change in heart rhythm or treatment for AF. The rest of outcomes, described in this aim, will be gathered after patient discharge from medical records and will be used to evaluate the clinical significance of the intervention.

Experimental Design: This is a pilot, single dose prospective, double-blind, placebo-controlled, randomized study among a placebo group and a Vitamin C 200mg/kg/day group (divided into four doses throughout the day). The dose was chosen based on preliminary data in sepsis patients. Patients will receive their first dose at the beginning of surgery prior to CPB so the myocardial tissue has uptake time prior to ischemia and reperfusion. The remaining three doses will be infused 6 hours apart. All patients will be operated-on by a limited surgical team.

Inclusion criteria	Exclusion criteria
1. Age ≥18	1. Low ejection fraction patients (<35%)
2. Patients undergoing non-emergent elective valve replacement or valve/CABG	2. Patients with autoimmune disease and those on immunosuppressant therapy
3. No known coagulopathy prior to surgery	3. Emergency
4. Patients with relatively well preserved	4. History of renal calculi, low urine output, renal

Inclusion criteria	Exclusion criteria
myocardial function (Ejection fraction >35%)	dysfunction (creatinine clearance < 40 ml/min or serum creatinine greater than 1.8mg/dl preoperatively) 5. Patients with known bleeding diathesis 6. Active infection or Active tumor 7. History of A-Fib 8. Glucose-6-phosphate deficiency 9. CABG only

Study Procedures: All patients will be approached prior to their surgery with the potential to participate in the study, and a formal Institutional Review Board (IRB) approved Informed Con-sent will be discussed, and all questions answered prior to obtaining their informed consent in writing. Their informed consent will be documented in the patient medical record, the clinical re-search office and all patients will receive copies of their signed consent forms. Once consented, patients will be randomized (computer generated) to receive either placebo, Normal Saline (NS) or Vitamin C. Experimental pharmacy will make up the infusion bags containing NS or sterile water for injection plus Vitamin C. The IV bags will be sent to the operating room and since Vitamin C has no color, the labeling of the bags would maintain this as a double blind study. Neither the clinicians caring for the patient nor the research (coagulation and inflammation) laboratory personnel would know the identity of the placebo vs. Vitamin C grouping of the patient. If a severe adverse event were to occur the clinicians could break the code by calling the experimental pharmacy. Anesthesia, perfusion (CPB) and surgery will be techniques in standard clinical usage by the anesthesia and surgery team. Enrollment in this study will not affect the standard care of the patients. Blood samples will be obtained prior to treatment (base-line- after anesthesia induction but prior to drug administration) and then throughout the 96 hour study (60 minutes on

CPB, 20 minutes after protamine, in ICU at 12, 48 and 96 hours, respectively). The total amount of blood drawn at each of the above time points will be approximately 15cc. With the 6 time points outlined that means an excess of 90cc of blood drawn above the usual clinical blood draws/loss of surgery. Other clinical data will be obtained from the patient's chart under direct supervision of the PI by a research coordinator and a graduate student.

5.4.4 Sample size calculation and statistical Analysis Data from this pilot human trial will be used for future development of a larger efficacy outcome trial. The effect of Vitamin C on in-flammation during cardiac surgery is a novel idea and often must start small due to cost and feasibility concerns.(193) Our study will enroll 24 to 50 patients with 12 patients in each arm of this safety and efficacy study in this new patient population. Descriptive statistics will be used to report patient demographics, medical histories which will allow for population description, identification of potential confounding factors and further hypothesis generation. Categorical variables will be reported as absolute and percent values. Continuous variables (such as number of vessels bypassed) will be expressed as median and interquartile ranges. Chi-square and Mann-Whitney tests will be used to compare categorical and continuous variables among the different groups using the JMPpro 10.0 Software for Windows. All tests will be two-sided using $\alpha=0.05$ level of significance. Distributions of all measures will be examined to identify possible outliers; outliers will be thoroughly checked for collection or data entry errors before being used in the analysis.

5.4.5 Ethical Aspects of Proposed Research This research will involve the use of fresh blood drawn from patients. The blood draws will be done, when possible from in-

dwelling arterial line and central line catheters and at the time of their routine blood draws. It is anticipated therefore that the only extra needle sticks a patient may have to endure would be at the 48 and 96 hours, which is most likely after removal of the arterial and central line catheters. The toxicity of Vitamin C is very low (discussed earlier), therefore the research team feels that the potential benefit far outweighs known risks. Patients will not be paid for participation and there will be no penalties imposed for either refusing to participate or withdrawing consent once enrolled.

5.4.6 Future research direction Data from this study will be used to power a larger clinical trial with a more inclusive test panel. If the hypothesis holds, Vitamin C effects as an anti-sludging agent to protect against blood stasis observed in the CPB machine will be explored.

5.4.7 Limitations of proposed research A small number of patients will be involved in this pilot, but this is usually encountered in feasibility studies to standardize the protocols as well as esti-mating the effects for sample size calculation. Only few biomarkers will be employed which may not capture the whole effects of the intervention, however this was compromised by just choos-ing those of highest relevant to cardiovascular conditions and for which association with clinical outcomes has been demonstrated.

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