NUTRITIONALLY-INDUCED OXIDATIVE STRESS AND VIRAL INFECTION

by

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ABSTRACT

WEI LI: Nutritionally-induced Oxidative Stress and Viral Infection

(Under the direction of Melinda A. Beck, Ph.D.)

This study was designed to investigate the effect of selenium (Se) or vitamin C deficiency on the immune response to infection with influenza virus. In the first part of the study, we tested whether Se deficiency would affect the immune response and subsequent lung pathology in mice infected with a virulent, mouse-adapted strain of influenza virus, influenza A/Puerto Rico/8/34. In the second part of the study, we tested whether the deficiency or supplementation of another important nutrient, vitamin C, could affect the immune response to influenza infection. Because mice can synthesize vitamin C, we utilized a gulonolactone knockout mouse *(gulo -/-)* model for our vitamin C deficiency studies.

There were no differences in lung influenza A/PR8/34 viral titers between the Se-adequate and the Se-deficient mice, or differences in lung influenza A/Bangkok/1/79 viral titers between the vitamin C-adequate and the vitamin C-deficient mice. However, vitamin C-deficient male mice had a higher lung viral titer when infected with the more virulent influenza A/PR8/34 compared with vitamin C-adequate mice. This difference was not found in the female vitamin C-deficient mice. In addition, the Se- and vitamin C-deficient mice had an altered immune response to influenza virus infection. Although vitamin C deficiency increased lung pathology late post infection in both male and female *gulo-/-* mice, there was

a clear sex difference in the effect of vitamin C deficiency on the immune response, as the difference in the expression of chemokines and cytokines was only observed in male vitamin C-deficient mice. This study demonstrated that a deficiency in either Se or vitamin C can alter the immune response to influenza virus infection, resulting in altered lung pathology. In addition, the sex differences found in the vitamin C-deficient mice suggest a further complexity in the response of the host to antioxidant nutrient deprivation. Clearly, an adequate Se and vitamin C intake is essential for a healthy immune response against infectious disease and other antioxidants cannot fully compensate for their deficiency.

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LIST OF ABBREVIATIONS

3' UTR	3' untranslated region
AA	ascorbic acid
AP-1	activator protein 1
APC	antigen presenting cell
DHA	dehydroascorbic acid
dsRNA	double-stranded RNA
EMSA	electrophoretic mobility shift assay
GM-CSF	granulocyte macrophage-colony-stimulating factor
GPX	glutathione peroxidase
GSH	glutathione
GSSG	glutathione disulfide
Gulo	L-gulono-γ-lactone oxidase
GAPDH	glyseraldehyde-3-phosphate dehydrogenase
HA	hemagglutinin
HAU	hemagglutinating unit
HDL	high density lipoproteins
ICAM 1	intercellular adhesion molecule 1
Influenza PR8	influenza A/Puerto Rico/8/34
IFN	interferon
IκB	inhibitory κB
IKK	IκB kinase

IL	interleukin
LDL	low density lipoprotein
M	matrix
MAPK	mitogen-activated protein kinase
MCP	monocyte chemotactic protein
MHC	major histocompatibility class
MoCM	monocyte conditioned medium
NA	neuraminidase
NF-κB	nuclear factor-κB
NIK	NF-κB inducing kinase
NK cells	natural killer cells
NP	nucleocapsid
PKR	protein kinase R
qRT-PCR	quantitative real time PCR
RNP	ribonucleoprotein
RNS	reactive nitrogen species
ROI	reactive oxygen intermediates
ROS	reactive oxygen species
RANTES	regulated upon activation normal T expressed and secreted
Se	selenium
SECIS	selenocysteine insertion sequence
SOD	superoxide dismutase
ssRNA	single-stranded RNA

Th1/2	T helper type 1/2
TLR	toll-like receptor
TNF	tumor necrosis factor
Trx	thioredoxin
TrxR	thioredoxin reductase
VLDL	very low density lipoproteins

Chapter I

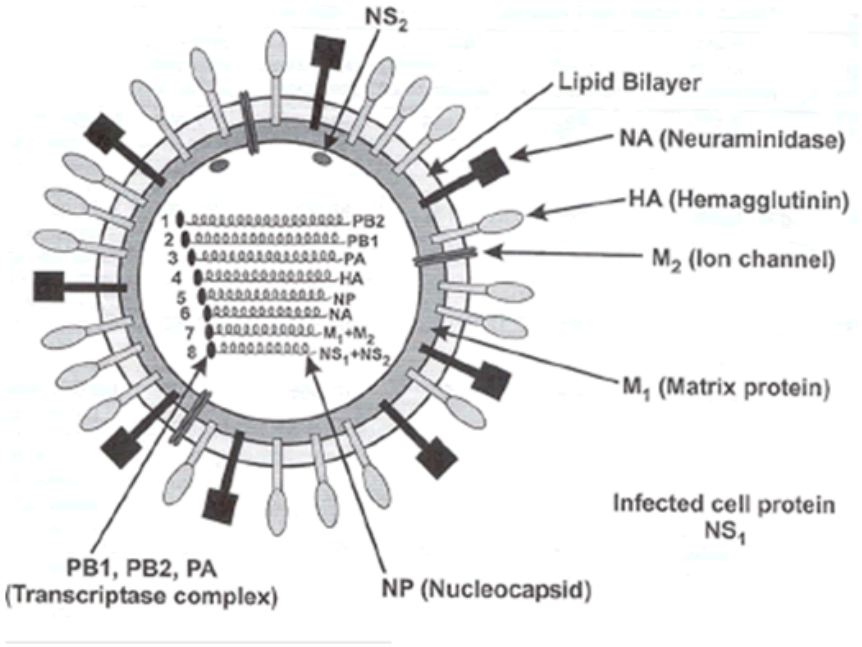
Introduction

A. Influenza virus

Influenza virus belongs to the virus family Orthomyxoviridae. Influenza viruses are divided into influenza A, B C, and thogotovirus (sometimes called influenza D virus) based on the antigenic differences between their nucleocapsid (NP) and matrix (M) proteins. Influenza A viruses are further divided into subtypes based on the antigenic nature of their hemagglutinin (HA) and neuraminidase (NA) glycoproteins.

Influenza viruses are enveloped viruses. The lipid envelope of influenza viruses is derived from the plasma membrane of the host in which the virus is grown. There is a layer of about 500 HA and NA spikes radiating outward (10-14 nm) from the lipid envelope. Influenza A, B and C viruses also encode another integral membrane protein, the M2, NB and CM2 proteins, respectively. The viral matrix protein (M1) underlies the lipid bilayer and associates with the ribonucleoprotein (RNP) core of the virus. Inside the virus are the RNP structures which contain eight different segments of single stranded RNA. The RNA is coated by NP protein subunits. RNP structures are also associated with the RNA-dependent RNA polymerase complex, which consists of three P (polymerase) proteins, PB1, PB2, and PA. The NS2 protein is associated with M1 protein and is essential for export of RNP complex from the nucleus during viral replication [1]. A schematic diagram of the structure of the influenza A particle is shown in figure 1.1.

Figure 1.1: A schematic diagram of the structure of the influenza virus particle.



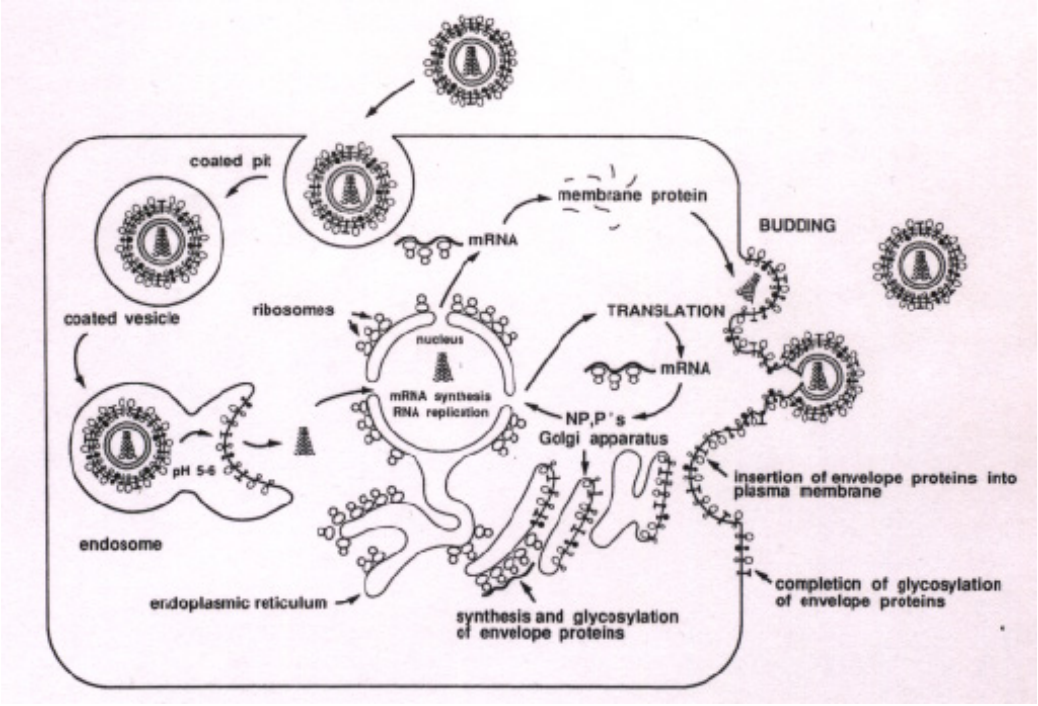
Modified from Lamb, RA and Krug, RM *Fields Virology* 4th ed: *Chapter 46.*

Influenza virus infection starts with the binding of HA molecules to sialic acid residues present on cell surface glycoproteins or glycolipids and the viruses enter cells by receptor mediated endocytosis. Influenza virus mRNA synthesis depends on host cell nuclear function. The virion associated polymerase uses capped RNA fragments derived by cleavage of host mRNA as primers for the initiation of viral mRNA synthesis [2]. Virus replication also requires an alternative type of transcription that results in the production of full-length copies of the vRNAs. The copying of vRNAs to template RNAs and the copying of template RNAs back to vRNAs do not require primers [3-6].

In polarized epithelial cells, influenza viruses assemble and bud at the apical surface of cells [7, 8]. HA , NA, M2 are synthesized on membrane bound ribosomes using viral

mRNA as templates and translocated to the apical surface [9-12]. Virons are assembled at the plasma membrane and bud from infected cells. A schematic diagram of the life cycle of influenza virus is shown in figure 1.2.

Figure 1.2: Schematic diagram of the life cycle of influenza virus.



Modified from Lamb, RA and Krug, RM *Fields Virology* 3rd ed: *Chapter 46.*

Infection with influenza virus causes a great deal of morbidity and mortality worldwide each year. In the U.S. alone, influenza virus infection results in over 36,000 deaths and 114,000 hospitalizations in a typical year [13]. During pandemics such as the one in 1918, a much greater loss of life occurs. The potential for the reemerging of highly pathogenic strains of influenza virus which are able to transmit from birds to humans has made influenza virus a dangerous threat.

B. Immune response to influenza virus infection

1. Innate immune response to influenza virus infection

The immune response to influenza infection has been well studied by utilizing animal models, as well as by studies in human populations. The influenza induced immune response is characterized by an initial innate immune response followed by an adaptive immune response. The immune response to influenza infection involves a coordinated response of a network of secreted proteins and activation of effector cells. Although the immune response can be divided into innate and adaptive responses, there is, in fact, considerable overlap. Many proteins secreted by the innate response are involved in activating and augmenting the adaptive response, and the adaptive response, in turn, can also enhance the innate response. The innate immune response acts immediately and does not require a prolonged period of induction. Cells involved in the innate immune response to influenza virus infection include epithelial cells, monocytes/macrophages, and natural killer (NK) cells.

Epithelial cells play an important role in the pathogenesis of influenza virus infection. When influenza virus begins to replicate in the epithelial cells of the respiratory tract and lungs, epithelial-cell-derived cytokines recruit and activate immune cells. The viral replicative intermediate double-stranded RNA (dsRNA) is critical for this process [14, 15]. Toll-like receptor-3 (TLR-3) and dsRNA-dependent protein kinase R (PKR) respond to dsRNA and activate nuclear factor-κB (NF-κB) [16-18]. The recognition of endosomal single-stranded RNA (ssRNA) by toll-like receptor-7 (TLR-7) also contributes to the stimulation of interferon- α/β (IFN-α/β) production [19]. Ultimately, an epithelial response is elicited which includes the secretion of the cytokines interleukin-8 (IL-8), interleukin-6 (IL-

6), regulated upon activation normal T cell expressed and secreted (RANTES), IFN-β and the up-regulation of the intercellular adhesion molecule-1 (ICAM-1) [18].

Monocytes/macrophages are among the very first cells to respond to influenza virus infection. Macrophages mature continuously from circulating monocytes that leave the circulation to migrate into tissues throughout the body, including the respiratory tract and the lungs. This recruitment of monocytes by extravasation into the infected tissue is a crucial event in the virus-induced inflammatory response and is mediated by lymphocyte attracting chemokines.

Monocytes/macrophages themselves are susceptible to influenza A virus infection. Within 24-48 hours, the infected monocytes die by apoptosis. Before cell death, infected monocytes initiate a cell-specific immune response, including the transcription and release of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), IFN-α, IFN-β, and CC chemokines (primarily RANTES, macrophage inflammatory protein-1 (MIP-1), and monocyte chemotactic protein-1 (MCP-1)). NF-κB and activator protein-1 (AP-1) are involved in the activation of transcription. Thus, infection of monocytes/macrophages with influenza virus primes for a rapid proinflammatory reaction and induces immigration of more monocytes/macrophages into infected tissue [20].

NK cells play a key role in the innate immune response against influenza virus infection by secreting interferon-γ (INF-γ) and by destroying virus infected cells. The cytotoxic activity of NK cells is controlled by two types of surface receptors: “activating receptors” and “inhibitory receptors”. Activating receptors recognize a wide variety of carbohydrate ligands present on many cells. In the case of influenza virus infected cells, the ligands could be viral proteins expressed at the surface of the infected cells. There is a direct

interaction between human NK cell NKp46 receptor and the hemagglutinin (HA) proteins present on influenza infected cells [21]. Inhibitory receptors prevent NK cells from killing normal host cells by recognizing major histocompatibility class I (MHC I) molecules normally found on all nucleated cells. However, cytotoxic T cells recognize virus-infected cells in conjunction with expression of MHC I. Influenza viruses, in an effort to evade detection by cytotoxic T cells, are able to down regulate MHC I expression in infected cells [22]. However, this viral strategy will render infected cells more vulnerable to NK cell killing. Upon detection, NK cells bind to infected cells and release cytotoxic granules. These cytotoxic granules contain effector proteins that penetrate the cell membrane and induce apoptosis.

The innate immune response to influenza virus infection involves an upregulation of antiviral IFNs, proinflammatory chemokines, and proinflammatory cytokines. The IFN-α/β-induced cellular antiviral response is the first line of defense against influenza virus infection by the host [23]. Type I IFNs, IFN-α and IFN-β are produced in direct response to a virus infection and consist of the products of the IFN-α multigene family, which are predominantly synthesized by leukocytes, and the product of IFN-β gene, which is synthesized by most cell types but particularly by fibroblasts and epithelial cells. The induction of type I interferon by influenza virus infection is mediated by the recognition of endosomal ssRNA by TLR7 [19]. Type II IFN consists of the product of the IFN-γ gene. Rather than being induced directly by viral infection, IFN-γ is synthesized in response to the recognition of infected cells by activated T lymphocytes and NK cells [24].

In addition to interferons, influenza infection induces the production of various proinflammatory chemokines and cytokines. Chemokines are potent chemoattractant

cytokines and have been considered the main candidate molecules responsible for the selective recruitment of distinct leukocyte populations. Member of the CC-chemokine subfamily, such as RANTES, MCP-1, and MIP-1α preferentially attract monocytes and lymphocytes [25]. RANTES is produced by CD8+ T cells, epithelial cells, fibroblasts and platelets and plays a key role in the immune response to viral infection by promoting leukocyte infiltration to sites of inflammation[26, 27]. MCP-1 has inflammatory properties similar to RANTES in terms of recruiting monocytes and lymphocytes. RANTES and MCP-1 are both involved in several inflammatory disorders of the lung [28, 29]. MIP-1α is produced by a variety of cell types, including monocytes, macrophages, mast cells, Langerhans cells, fibroblasts, and T cells. MIP-1α is primarily chemotactic for B cells, activated CD8+ T cells, NK cells, and eosinophils [30-33]. MIP-1α and MIP-1β together stimulate mature tissue macrophage proliferation and MIP-1α alone stimulates the secretion of TNF, IL-1α, and IL-6 by peritoneal macrophages [34]. MIP-1α also increases cell adhesion by inducing ICAM-1 expression [35]. Influenza virus infected MIP-1α knockout mice have significantly less lung pathology compared with MIP-1α wild-type controls, suggesting that MIP-1α plays an important role in the inflammatory response to influenza virus infection [36].

Proinflammatory cytokines that are produced in respond to an influenza virus infection are interleukin-12 (IL-12), IL-1β, and TNF-α. IL-12 is produced by monocytes, macrophages, dendritic cells, neutrophils, and to a lesser extent, B cells. The major actions of IL-12 are on T and NK cells. IL-12 induces proliferation, IFN-γ production and increased cytotoxic activity of these cells, and importantly, IL-12 induces the polarization of CD4+ T cells to a T helper type 1 (Th1) phenotype that mediates immunity against intracellular

pathogens. IL-12, especially in combination with IL-18, also acts on macrophages and dendritic cells to induce IFN-γ production [37].

IL-1β is a primary regulator of the inflammatory response. IL-1β causes leukocyte accumulation by inducing adhesion receptors on vascular endothelium and stimulating chemokine production. IL-1β also induces the production of other cytokines, prostanoids and nitric oxide. It stimulates hepatic acute-phase protein synthesis, acts as an accessory signal for lymphocyte activation and is the major endogenous pyrogen [38]. TNF-α is also a pleiotropic cytokine with a diverse range of biological activities. The principal TNF-α producing cells are monocytes and macrophages, and additional producers include B, T and NK cells. The release of TNF-α induces a local protective effect. TNF-α acts on blood vessels to increase vascular permeability to fluid, proteins, and cells, and to increase endothelial adhesiveness for leukocytes and platelets. TNF-α also stimulates an acute-phase response in the liver [39].

2. Adaptive immune response to influenza virus infection

The innate immune response to influenza infection is followed by the adaptive immune response characterized by the generation of antigen-specific effector cells that specifically target the pathogen and development of memory cells that can prevent reinfection with the same or similar pathogen [39]. The adaptive immune response involves the antigen-specific activation and proliferation of B and T cells and antigen presenting cells (APCs) that display antigen to B and T cells. Together they respond to the foreign antigen [39].

During influenza infection, viral peptides are presented on the surface of APCs, primarily dendritic cells. Dendritic cells reside in most tissues, including the lungs and

epithelial cells of the respiratory tract. After phagocytosing viral particles at the site of infection, dendritic cells migrate from the infected tissue to draining lymph nodes, where they initiate the adaptive immune response to influenza virus. CD8+ T cells are activated by dendritic cells presenting viral antigen in the context of MHC I and CD4+ T cells are activated by dendritic cells presenting viral pathogen in the context of MHC II.

Influenza virus-infected dendritic cells stimulate strong proliferative and cytolytic responses from human CD8+ T cells [40]. Influenza specific CTLs play an important role in eliminating influenza virus infected cells by recognizing and lysing those cells presenting influenza antigen [41]. In mice lacking CD8+ cells, infection with influenza A/PR/8/34 (PR8) led to increased viral replication and eventual mortality [42]. CD4+ cells can be divided into Th1 or Th2 classes, depending on the panel of cytokine they secrete. Th1 responses enhance CD8+ T cells, whereas Th2 responses are involved in providing help for B cell activities. Viral infections are known to predominantly induce the Th1 type response that promotes the activation of CD8+ T cells and macrophages and drives B cell differentiation [43]. The Th1 response to influenza virus infection has also been shown to inhibit the development of Th2 cells [44]. CD4+ depleted mice can effectively clear PR8, implying that CD4+ cells are not required for elimination of virulent influenza strains [45]. However, when both B cells and CD4+ T cells are absent, mice can not efficiently clear influenza PR8 infection and have a high mortality rate [45], suggesting that viral clearance requires both B and T cells. Mice lacking both functional CD8+ T cells and B cell could not clear even a mild strain of influenza virus [46], indicating that CD4+ cells alone can not maintain sufficient anti-influenza immunity and CD4+ cells may only participate in the immune response to influenza virus indirectly by providing help for CD8+ cells and B cells. B cells also play an

important role in immunity to influenza. Clearance of PR8 was compromised in B cell-deficient mice. However, unlike CD8+ T cells, B cells are not absolutely required if the infection is caused by a mild strain of influenza virus [47]. A summary of the efficacy of different combinations of CD8+, CD4+, and B cells in clearing influenza virus is shown in **table 1.1** [43, 48].

Table 1.1: The efficacy of different combinations of CD8+, CD4+, and B cells in clearing influenza virus. For a virulent strain of influenza, neither CD8+ cells, CD4+ cells, nor B cells alone can effectively clear virus and mice succumb to what would otherwise be a sublethal dose of virus. However, if combinations of cells are present, mice can clear the infection with slightly delayed kinetics and increased survival. Therefore, the immune response to pathogenic strains of influenza requires a complex interplay between cytotoxic T cells, antibody secreting B cells and cytokine secreting CD4+ cells.

CD8	CD4	B cells	Clearance (days)	Survival (%)
+	+	+	7-10	100
-	+	+	10-14	100
-	+	-	>20	0
-	-	+	>20	0
+	-	-	>14	20
+	+	-	10-14	35-85
+	-	+	10-14	90
-	-	-	>20	0

Each cell type of the adaptive immune response was depleted via treatment with antibody, using genetically altered mice, or a combination of both.

Modified from Brown, D.M. et al. *Seminars in Immunology* (2004). 16(3), 171-177.

The adaptive immune response to influenza infection also generates immunological memory to influenza virus. Both T and B memory cells respond differently to an antigen compared with the primary response to the same antigen. In the case of T cells, memory CD8+ T cells are more sensitive to restimulation by antigen than are naive cells and more quickly and vigorously produce cytokines such as IFN-γ in response to stimulation [39]. CD8+ effectors and resting memory CD8+ cells promote host recovery during lethal pulmonary influenza virus infection, while similar frequencies of naive CD8+ cells are not protective upon adoptive transfer [49]. Only after re-exposure to antigen do memory CD4+ T cells achieve armed effector T-cell status and secrete type 1 or type 2 cytokines respectively [39]. Similar with memory T cells, antigen-specific memory B cells also differ both quantitatively and qualitatively from naive B cells in the type of antibody produced and the intensity of the response [39].

3. Cytokines bridge the innate and adaptive immune responses

The nonspecific responses of innate immunity are necessary for an adaptive immune response to be initiated and together they work to form a complete immune response. The innate immune response produces changes in the immediate environment and sets the stage for the adaptive immune response. These include the release of inflammatory cytokines, which will regulate the adaptive immune response, and the activation of APCs, which will later present antigens to T-cells and B-cells.

The adaptive immune response is affected profoundly by IFNs. All IFN family members share the ability to enhance the expression of MHC class I proteins and thereby to promote CD8+ T cells responses. In contrast, only IFN-γ is capable of inducing the expression of MHC class II proteins, thus promoting CD4+ T cell responses [24]. IFN-γ also

plays an important role in regulating the balance between Th1 and Th2 cells. IFN-γ stimulates the synthesis of IL-12 by APCs [50-52], which drives CD4+ cells to become Th1 cells [53, 54]. On the other hand, IFN-γ inhibits IL-4 production, preventing the development of Th2 cells [55, 56]. IFN-α and IFN-β also play a role in stimulating the adaptive immune response. IFN-induced IL-15 can stimulate the division of memory T cells [57]. IFN-α and IFN-β appears to be able to promote the survival of activated T cells directly [58]. In addition to regulating T cell development and survival, IFNs also have direct effects on B cell development and proliferation, immunoglobulin secretion and immunoglobulin heavy-chain class switching [24].

CC chemokines RANTES and MIP-1α have been correlated with a Th1 type immune response [59-62]. CC chemokine receptor 5 (CCR5), the shared receptor for MIP-1α/β and RANTES, is selectively expressed by Th1 cells and virtually absent from Th2 cells [61-64]. During influenza virus infection, the upregulation of CC chemokine levels as part of the innate immune response predisposes the adaptive immune response to the Th1 type.

C. Oxidative stress alters the function of the immune system

1. Oxidative stress enhances inflammatory response to infection

Inflammatory responses to infection must be precisely regulated to facilitate microbial killing while limiting host tissue damage. The generation of an immune response involves the activation of effector cells such as phagocytes, lymphocytes and NK cells, as well as subsequent production of cytokines and other mediators, mainly reactive oxygen species (ROS). Neutrophils and other phagocytes manufacture the highly reactive O_2^- by NADPH oxidase at the expense of NADPH. Cells contain superoxide dismutase (SOD) in order to deal with the toxic O_2^- radicals, creating H_2O_2 in the process. Because H_2O_2 can also be toxic to cells, cellular enzymes such as glutathione peroxidase and catalase breakdown H_2O_2 to less reactive species. Although the ROS can be used by phagocytic cells to aid in microbial killing [65], these oxidants can also cause direct damage to cellular structures. In addition, ROS production can be involved in subsequent morbidity and mortality due to excessive activation of immune cells during an infectious processes [66]. Changing the redox environment in which the immune system operates towards an increased oxidizing environment has been shown to lead to a hyperresponsive innate immune system and to enhanced activation of the adaptive immune responses involving APC maturation and T cell activation [67].

2. ROS participate in cellular signaling and transcriptional regulation

There is considerable evidence implicating the role of ROS in cellular signaling and transcriptional regulation [68, 69]. NF-κB has been considered a redox-sensitive transcription factor [70]. The Rel/NF-κB family of transcriptional factors regulate expression of numerous

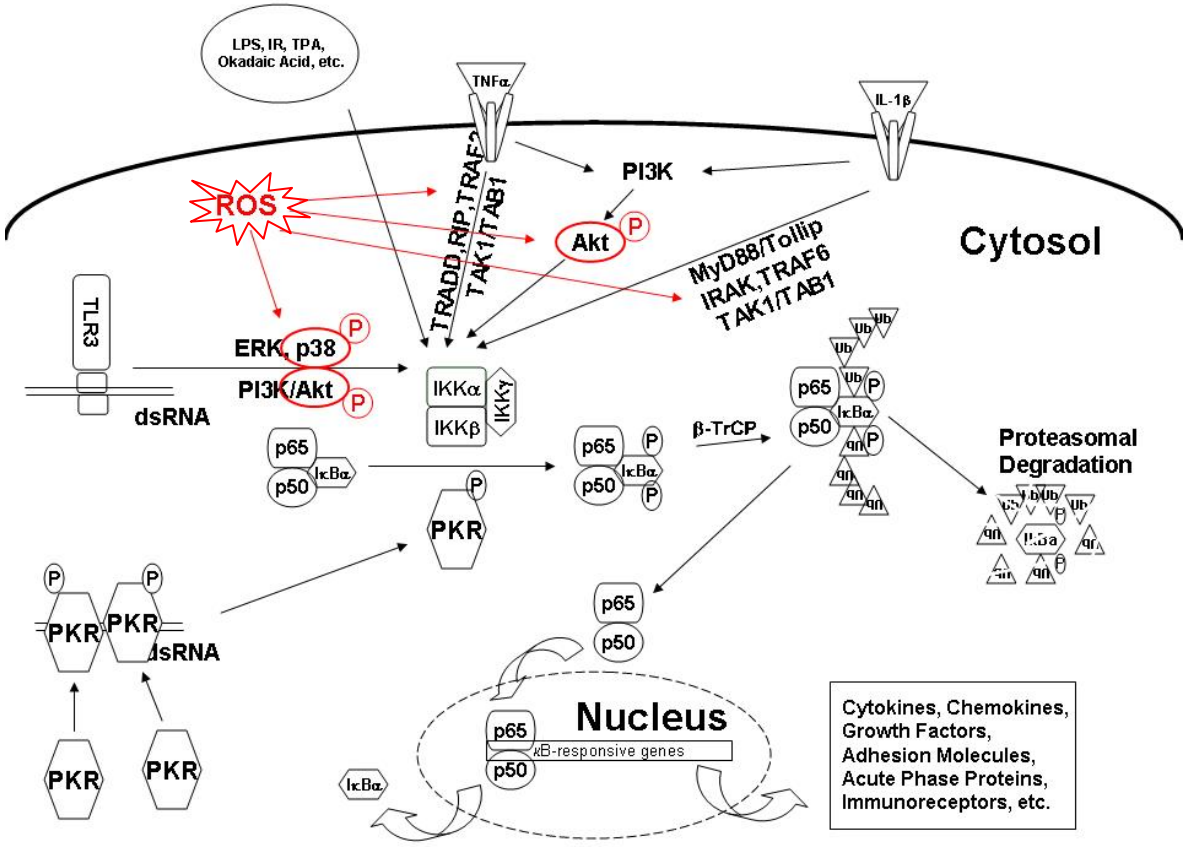
cellular and viral genes and play important roles in immune and stress responses, inflammation, and apoptosis [71-74]. NF-κB is a dimeric eukaryotic transcription factor which is activated by a variety of inflammatory stimuli, including cytokines and viral infection [73, 75]. In unstimulated cells, the inactive form of NF-κB is retained in the cytosol by binding to the inhibitory factors known as IκB family of inhibitory proteins. Upon stimulation, IκBs are rapidly phosphorylated and degraded via proteasomal pathways. The degradation of IκBs releases NF-κB, allowing transport to the nucleus where they will bind to the NF-κB response elements on DNA and regulate gene transcription. The NF-κB-inducing kinase (NIK) activates NF-κB by inducing the phosphorylation of IκB. The downstream IκB kinase complex (IKKα, IKKβ) phosphorylates IκBα directly. The multiple subunit IKK is activated by phosphorylation in response to inflammatory signals.

Toll like receptor 3 (TLR3) and PRK play important roles in NF-κB activation during influenza virus infection. Double-stranded RNA (dsRNA) is produced by most viruses during their replication [15]. Mammalian TLR3 recognizes dsRNA and activation of the receptor induces the activation of NF-κB [17]. TLR3 expression is up-regulated either by influenza A virus or by purified dsRNA but not by other major inflammatory mediators; and influenza A virus and dsRNA induce epithelial cell activation through MAPK p38, PI3K/Akt signaling [18]. PKR is activated by dsRNA by a mechanism involving autophosphorylation. The binding of two molecules of monomeric PKR with a single dsRNA molecule leads to dimerization and autophosphorylation. Phosphorylated PKR is independent of dsRNA and phsophorylates IκB [16].

In addition to inflammatory cytokines and viral proteins, oxidative stress also activates NF-κB [70]. H_2O_2 treatment activates NF-κB by increasing the phosphorylation of

mitogen-activated protein kinase (MAPK) p38 and Akt, which are factors of the protein kinase cascades leading to IκB phosphorylation [69]. Both MAPK p38 and Akt are intermediates in the signaling from TLR3-dsRNA recognition to NF-κB activation, implying that oxidative stress may be able to amplify the effect of dsRNA on NF-κB activation in a synergistic manner. Enhanced activation of NF-κB would lead to increased expression of inflammatory cytokines, chemokines, immunoreceptors, cell adhesion molecules and MHC I [24], which will cause a hypersensitive immune response, leading to increased lymphocyte infiltration and tissue damage. A schematic diagram of NF-κB activation by proinflammatory cytokines, dsRNA, and ROS is shown in figure 1.3. The target genes of NF-κB have been reviewed in detail [76].

Figure 1.3: NF-κB activation by cytokines, dsRNA, and ROS. Oxidative stress increases the phosphorylation of MAPK p38 and Akt, which mediate NF-κB activation by dsRNA and cytokines. The presence of influenza virus alone with oxidative stress may lead to a hypersensitive immune response.



D. Selenium

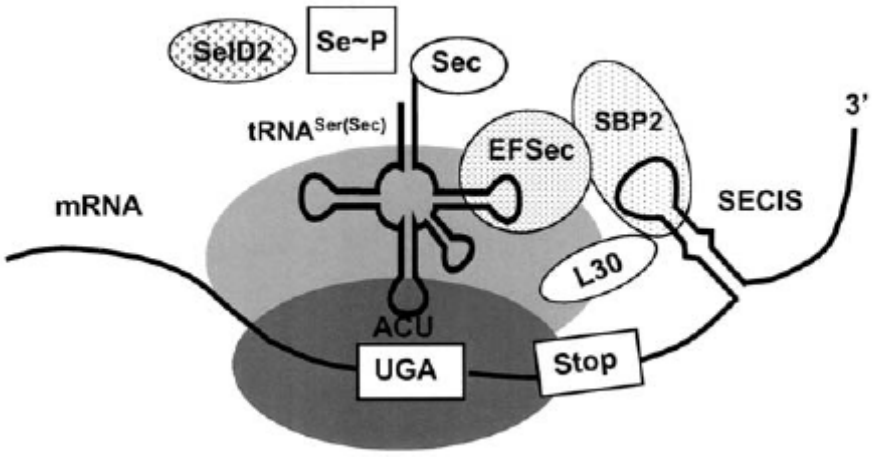
1. Chemistry and biological function

Selenium is of fundamental importance to human health. It is an essential component of several major metabolic pathways, including thyroid hormone metabolism, antioxidant enzyme defense systems, and immune function. Selenium is incorporated as selenocysteine at the active site of a wide range of selenoproteins. The Se in selenocysteine is an extremely efficient biological catalyst [77]. Currently, about 25 selenoproteins have been discovered in humans [78].

In prokaryotes, archaebacteria, and eukaryotes, selenocysteine is encoded by a UGA codon [79]. In prokaryotes, the incorporation of selenocysteine is with the help of $tRNA^{Sec}$ (SelC), Sec-specific elongation factor (SelB), and a cis-acting mRNA structure, selenocysteine insertion sequence (SECIS). In addition, selenocysteine synthase (SelA) and selenophosphate synthetase (SelD) are involved in the biosynthesis of selenocysteine on the $tRNA^{Sec}$. Homologs of the prokaryotic genes have been identified in eukaryotes [80] and the mechanism for selenocysteine incorporation is partially conserved between the two kingdoms. However, the eukaryotic selenoprotein biosynthetic pathway has evolved several unique features that provide a higher level of complexity and greater potential for regulation [81]. In eukaryotes, selenocysteine is synthesized by ATP-dependent phosphorylation of serine loaded on its $tRNA^{Sec}$ (SelC gene homolog). This reaction is catalyzed by selenophosphate synthase 2 (SelD2), which is a Se-dependent Sec-containing enzyme by itself, suggesting a possible feed-back regulation mechanism. Cotranslational incorporation of selenocysteine into selenoporteins requires recruitment of a selenocysteine-specific transcription factor EFSec (SelB gene homolog) to the ribosome, which replaces the normal elongation factor

(EFtu) and prevents binding of release factors to the UGA codon. In eukaryotes, recognition of the selenocysteine UGA codon is assisted by a SECIS binding protein (SBP2), which is essential for selenocysteine incorporation *in vitro* [82]. Unlike the SECIS of prokaryotes, which is located immediately downstream of the selenocysteine UGA codon [83], the eukaryotic SECIS element is located in the 3' untranslated region (3' UTR) of the mRNA [84]. Eukaryotic selenoprotein synthesis is shown in figure 1.4.

Figure 1.4: Eukaryotic selenoprotein synthesis. Cotranslational incorporation of selenocysteine is encoded by the UGA stop codon in the selenoprotein mRNAs. A hairpin-loop structure (SECIS element) in the 3'-untranslated region of these mRNAs binds selenocysteine binding protein (SBP2), which interacts with the elongation factor EFSec selective for binding $tRNA^{Sec}$. The protein L30 is another factor involved in the processive interaction between SBP2 and the ribosome. The biosynthesis of selenocysteine occurs on the seryl-loaded $tRNA^{Ser}$. This step requires ATP-dependent formation of selenophosphate Se~P, which is catalyzed by the selenoprotein selenophosphate synthase (SelD2) phosphorylating reduced selenide and allowing modification of $tRNA^{Ser}$ to yield $tRNA^{Sec}$.



Modified from Josef Kőhrle, Thyroid (2005), 15(8), 841-853.

The glutathione peroxidases (GPXs) represent a major class of functionally important selenoproteins. GPXs catalyze the GPH-dependent reduction of hydrogen peroxide and organic hydroperoxides and thus provide protection of the cells from oxidative damage. Five

selenocysteine-containing GPXs have been detected: the cytosolic or classical GPX1, gastro-intestinal GPX2, plasma GPX3, phospholipids hydroperoxide GPX4 and GPX5, which is only present in the sperm nuclei [85]. The membrane-bound phospholipids hydroperoxide GPX4 (PHGPX) detoxifies phospholipid hydroperoxides and along with vitamin E, helps prevent oxidative damage to membranes. GPX4 may be more important than GPX1 in protecting the cell from oxidative stress. Plasma GPX3 eliminates peroxide in the extracellular fluid. In addition, the GPXs play a vital role in the synthesis of arachidonic acid metabolites. The lipoxygenase and cyclooxygenase pathways produce hydroperoxyeicosatetraenoic acids, which must be reduced for lipoxin, prostaglandin and leukotriene synthesis [86]. Eicosanoid synthesis is depressed in Se deficiency [86].

Mammalian thioredoxin reductases (TrxRs) are another family of seleno-containing enzymes. They also contain selenocysteine as the penultimate C-terminal amino acid residue [87], which is indispensable for their enzymatic activity [88]. TrxRs catalyze the NADPH-dependent reduction of oxidized thioredoxin. Reduced thioredoxin is a central factor in cellular redox regulation. It provides reducing equivalents for various redox-dependent systems, e.g. for ribonucleotide reductase essential for DNA synthesis and for the redox regulation of transcription factors, and has important functions in regulating cell growth and inhibiting apoptosis [89]. In addition to thioredoxin, mammalian TrxRs are able to use other substrates, including hydroperoxides, dehydroascorbate, and various enzymes and proteins [90]. This broad substrate specificity has been attributed to the presence of selenocysteine situated in the flexible C-terminal extension [88]. The TrxRs that have been detected include cytosolic TrxR1 [91], mitochondrial TrxR2 [92-95], and TrxR3, which is preferentially expressed in the testis [96]. Other studies suggest the existence of further thioredoxin

reductase species, which may differ with regard to their distribution among tissues and subcellular compartments and may have specific biological roles [85].

Thyroid hormone synthesis, metabolism and action require adequate availability of iodine as well as selenium [97]. The deiodinase enzyme family has three selenocysteine-containing iodothyronine deiodinases (D1, D2, D3) that contain selenocysteine in their active site [98-104]. Although mild Se deficiency does not affect expression of deiodinases in humans, Se supply may affect serum TH levels in individuals residing in regions with limited Se supply [97].

Other selenoproteins include selenophosphate synthetase 2, selenoprotein P, selenoprotein W, 15-kDa selenoprotein, 18-kDa selenoprotein, and those selenium-containing proteins not yet fully identified. The possible functions of these selenoproteins have been reviewed [85].

2. Food sources, absorption, transportation and metabolism

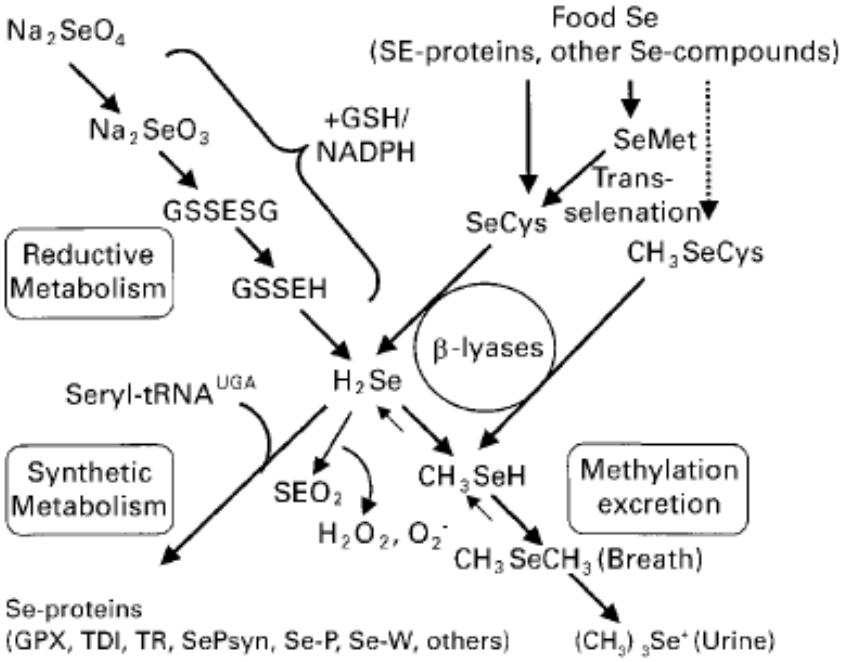
Selenium is present in most foods [105, 106]. However, human daily intakes vary significantly among different geological regions because of the variation in the selenium content of soils. Se is covalently bound into multiple compounds; those of biological importance include Se salts, Se derivatives of sulfur amino acids, and methylated derivatives of selenoamino acids. A schematic diagram of selenium metabolism pathways is shown in figure 1.5.

The destination of dietary Se is partially determined by its chemical form. Salts such as selenite and selenate and the amino acid selenocysteine are easily incorporated into selenoproteins, but since selenoprotein synthesis is tightly regulated, Se from these sources will not accumulate in the body beyond a certain point. However, selenomethionine

substitutes for methionine in proteins and accumulates in large protein pools such as muscle. Total Se body burden is much higher for selenomethionine than for selenocysteine or inorganic Se salts [107].

Laboratory experiments, clinical trials, and epidemiological data suggest that Se is a cancer preventive nutrient. The intermediates in the Se methylation pathway, including methylselenol (CH_3SeH) and dimethylselenide ($[CH_3]_2Se$), have been shown to have anticarcinogenic activity, while the fully methylated form, trimethylselenonium ($[CH_3]_3Se^+$), is totally ineffective [108, 109]. Since most of the Se cancer preventive studies have used Se levels far above the dietary requirement, levels at which the antioxidant enzymes GPX and TrxR activities have been saturated, the anticarcinogenic effect of Se was thought to be independent of its antioxidant activity. However, recent evidence showing an association between Se, reduction of DNA damage and oxidative stress together with data showing an effect of selenoprotein genotype on cancer risk implies that selenoproteins, especially the antioxidant selenoproteins are indeed implicated [110]. A number of mechanisms have been proposed to explain the anticarcinogenic effect of Se, including reduction of oxidative stress, hence reduction of DNA damage [111-113], reduction of inflammation [114], induction of carcinogen detoxification [115], enhancement of the immune response [116-118], and an increase in tumor-suppressor protein p53 [119]. Possible mechanisms for the anticarcinogenic effects of Se were recently reviewed in detail [119].

Figure 1.5: Pathways of Se metabolism. Oxidized inorganic Se forms (selenate, selenite) undergo reductive metabolism yielding hydrogen selenide (H_2Se), which is incorporated into selenoproteins co-translationally through modification of tRNA-bound serinyl residues at certain loci encoded by specific UGA codons. Successive methylation of H_2Se detoxifies excess Se, yielding methylselenol (CH_3SeH), dimethylselenide ($[CH_3]_2Se$) and trimethylselenonium ($[CH_3]_3Se^+$); the latter two metabolites are excreted in breath and urine respectively. Food protein can contain selenomethionine (SeMet) which can be incorporated non-specifically into proteins in place of methionine, and selenocysteine (SeCys) which is a product of SeMet catabolism and is itself catabolized to H_2Se pool by a β-lyase. Another lyase releases CH_3SeH from Se-methylselenocysteine (CH_3SeCys) present in some foods (e.g. *Allium* vegetables). Oxidation of excess H_2Se leads to production of superoxide and other reactive oxygen species.



Modified from Gerald F. Combs Jr, *British Journal of Nutrition* (2001), 85, 517-547.

Approximately 60% of Se in plasma is incorporated in selenoprotein P which contains 10 Se atoms per molecule as selenocysteine [120]. Extracellular GPX (GPX3), and selenoprotein P account for over 90% of plasma selenium and both may serve as a transport protein for Se. However, selenoprotein-P is also expressed in many tissues and has been associated with cell membranes [121] which suggests that it may also serve as an antioxidant [122].

3. The effect of selenium on the immune function and response to infectious diseases

Although the mechanisms involved have yet to be fully elucidated, it is well established that dietary Se is important for a healthy immune response [123]. Se influences both the innate and the adaptive immune systems [124, 125]. The effects of Se deficiency include reduced T-cell numbers, and impaired lymphocyte proliferation and function [117]. Supplementation of Se appears to boost immune cellular immunity by three mechanisms. First, it upregulates the expression of the T-cell high-affinity IL-2 receptor and provides a vehicle for enhanced T-cell responses [118]. Because the T cell is a key component in providing B-cell help for antibody synthesis, it may explain the stimulatory effects of Se on antibody production. Second, it prevents oxidative-stress-induced damage to immune cells. Third, it alters platelet aggregation by decreasing the ratio of thromboxane to leukotriene production [126]. The diverse effects of Se on immune cell function is listed in Table 1.2 (adapted from the review by McKenzie [126]).

Table 1.2: Effect of Se on Immune Function

Se supplementation

In vivo

↑ Neutrophil migration and O_2^{2-} activity (cow);

↑ High affinity IL-2 receptor (mouse);

↑ T-cell proliferation and function following age-related decline (mouse);

↑ Natural killer cell activity (mouse, human);

↑ Cytotoxic T-cell activity (mouse);

↑ T-cell response to pokeweed mitogen (cow);

↑ Lymphokine-activated killer cell activity;

↑ Delayed-type response due to better antigen presentation (mouse);

↓ Cell death following paraquat exposure (rat);

↓ UV induced skin cancers and mortality (mouse);

↓ Erythema following UV exposure (human, mouse);

↑ Vaccine-induced immunity to malaria (mouse);

In vitro

↓ HIV long-terminal-repeat activation and HIV replication in T-cell (human);

↓ NFκB activation (human);

↓ B-cell lipoxygenase activity (human);

↑ Antibody responses (primary and secondary) to virus (cow);

↓ Cell death following UV radiation to skin cells (mouse, human);

↓ DNA damage and lipid peroxidation in UV-exposed skin cells (mouse, human);

↓ IL-6, IL-8 and TNF mRNA following UV treatment of skin cells (human);

↓ Cell death following paraquat exposure (human);

↑ Apoptosis in tumors (mouse, human);

↓ Apoptosis induced by UV in normal skin cells (human);

↑ Phytohaemagluttinin response in lymphocytes (human);

↑ Killing by macrophages (human);

↑ Target killing by cytotoxic T cells (human);

Se deficiency

↑ Platelet aggregation and leukotriene synthesis (atopic human);

↓ IgG and IgM titres (human);

↓ Antibody production by lymphocytes (mouse);

↑ Virulence of coxackievirus (mouse);

↑ Virulence of influenza virus (mouse);

↓ Neutrophil chemotaxis (goat);

↓ Neutrophil and leukocyte activity (pig);

↓ Candidacidal activity by neutrophils (rat);

↑ CD4+ T cells, ↓ CD8+ T cells, ↓ CD4-/CD8- thymocytes (mouse).

↑ Secretion of poliovirus vaccine mutations (human);

Modified from McKenzie, R.C. et al. *Immunol Today,* 1998. **19**(8): p. 342-5.

Se is a key modulator of NF-κB activation. NF-κB is a redox-sensitive transcription factor that can be activated by ROS [70]. The direct evidence comes from stimulating tissue culture with H_2O_2 in vitro. However, this H_2O_2-induced NF-κB activation is highly cell type dependent and therefore H_2O_2 is unlikely to be a general mediator of NF-κB activation. A possible explanation for this cell type variation is that the activation of NF-κB by ROS is mediated by intracellular reduced glutathione (GSH), whose level differs from one cell type to another [70]. Intracellular thiol (mainly GSH) levels regulate the activation of NF-κB and mechanisms that control GSH levels also regulate those genes whose expression is dependent on the activation of NF-κB [127]. Since GSH exerts its antioxidant effect through the activity of GPX, the low GPX activity caused by Se deficiency may lead to NF-κB overactivation. Conversely, Se supplementation will reduce NF-κB activation upon stimulation. In the human hepatoma cell line HuH-7, NF-κB activation induced by monocyte conditioned medium (MoCM) and TNF-α is inhibited by Se at the physiological level (1.5 μmol/L) [128]. Over-expression of GPX suppresses NF-κB activation [129, 130], and this effect was not

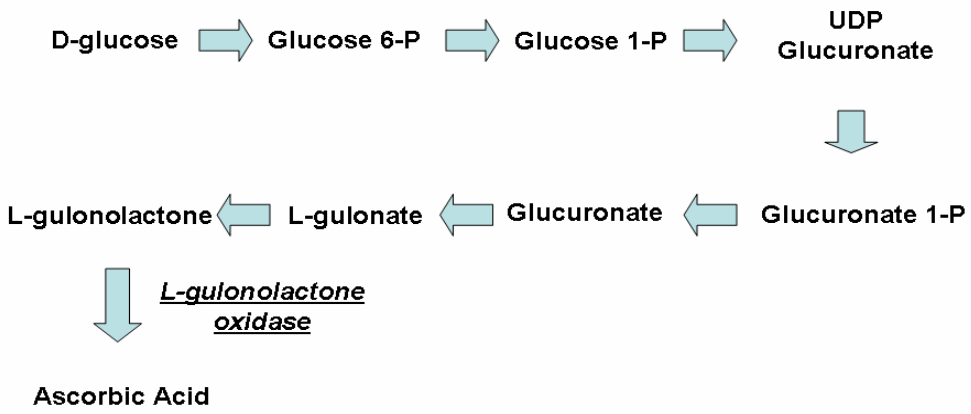
observed when the cells were deprived of Se [130], confirming that Se is a key modulator of NF-κB activation. Because Se is essential for thioredoxin reductase activity, Se also modulates NF-κB activation by affecting thioredoxin (Trx) levels in the cell. High Trx levels may keep IκB in its reduced form and prevent its phosphorylation. The cysteine residue(s) of NF-κB might be involved in the DNA-recognition by NF-κB and the redox control mechanism mediated by Trx may have a regulatory role in the NF-κB-mediated gene expression [131].

E. Vitamin C (Ascorbic acid)

1. Chemistry and biological function

Vitamin C is a six-carbon lactone that is synthesized from glucose in the liver by most mammalian species (figure 1.6), but not by humans, non-human primates and guinea pigs. These species do not have the functional enzyme, gulonolactone oxidase, which is essential for synthesis of the ascorbic acid immediate precursor 2-keto-1-gulonolactone. The DNA encoding for gulonolactone oxidase has undergone substantial mutation, resulting in the absence of a functional enzyme [132, 133].

Figure 1.6: Ascorbic acid synthesis



Modified from *Advanced human nutrition*, Robert E.C. Wildman and Dennis M. Medeiros, CRC Press, 2000

Vitamin C is a necessary cofactor for a number of enzymes, including prolylhydroxylase, lysylhydroxylase, trimethyllysine dioxygenase, 4-butryobetaine hydroxylase, phenylalanine mono-oxygenase, p-hydroxyphenyl pyruvate hydroxylase, dopamine mono-oxygenase and peptidyl glycine alpha amidating mono-oxygenase. Vitamin C is necessary to maintain the enzyme prolylhydroxylase in an active form by keeping its iron atom in a reduced state. Scurvy is caused by vitamin C deficiency that results in an underhydroxylation of proline and lysine in collagen.

Vitamin C is also an antioxidant. It prevents other compounds from being oxidized. The species that receive electrons and are reduced by vitamin C can be divided into several classes: 1) compounds with unpaired electrons (radicals) such as oxygen related radicals (superoxide, hydroxyl radical, peroxyl radicals), sulfur radicals and nitrogen-oxygen radicals. 2) Compounds that are reactive but are not radicals, including hypochlorous acid, nitrosamines and other nitrosating compounds, nitrous acid related compounds and ozone. 3) Compounds that are formed by reaction with either of the first two classes and then react with vitamin C. An example is the formation of the alpha tocopheroxyl radical, which is generated when exogenous radical oxidants interact with alpha tocopherol in low density lipoprotein (LDL). The tocopheroxyl radical can be reduced by ascorbic acid back to alpha tocopherol [134]. 4) Vitamin C participates in transition metal-mediated reactions by reducing transion metal ions [135-137].

However, caution should be exercised since vitamin C can exert pro-oxidant effects in vitro, usually by interaction with transition metal ions [138]. Vitamin C can reduce transition metals (Fe^{3+} to Fe^{2+}, Cu^{2+} to Cu^{1+}) and the reduced metals catalyze the formation of free radicals including the hydroxyl radical which can initiate peroxidative chain reaction.

Vitamin C should not be taken together with transition metals or be taken in iron overloaded conditions (e.g. haemochromatosis). Because a high vitamin C intake also induces the hepatic cytochrome P4502E1 isomers that are responsible for metabolizing ethanol to highly reactive radicals, care should be taken in alcohol drinkers [139].

2. Food sources, absorption, transportation, and tissue distribution

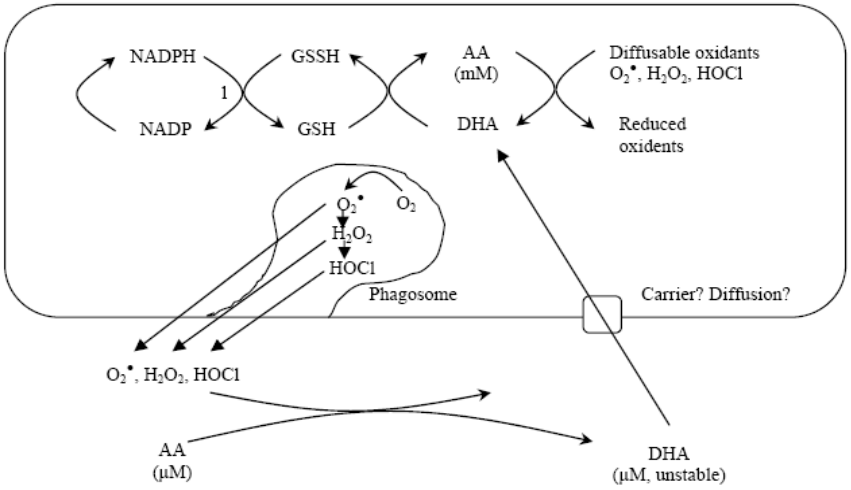
Vitamin C is mainly found in fruits and vegetables. The majority form (80-90%) of ascorbate in food is in its reduced form [140]. Vitamin C concentrations in plasma are tightly controlled [141, 142]. At plasma concentrations less than 4 μM, symptoms of scurvy may occur. Ascorbic acid is present in the blood at concentrations of 5-90 μmol/L in normal individuals [141]. Asorbate in plasma and serum is available to tissues and cells transporters directly without a protein-bound intermediate [143, 144]. Control of vitamin C concentrations is mediated by tissue transport, absorption and excretion. Ascorbate is accumulated in mmol/L concentrations in neutrophils, lymphocytes, monocytes, and platelets [141, 145-150], suggesting it may be important for the immune system.

3. The effect of vitamin C on immune function and response to infectious diseases

The mechanisms whereby vitamin C affects the immune system are poorly understood, although there is evidence indicating that it might affect, for example, functions of phagocytes, proliferation of T lymphocytes, and production of interferons and monocyte adhesion molecules [151-155]. So far, however, vitamin C has not been specifically linked to any single immunological mechanism. It is possible that vitamin C has nonspecific protective effects on diverse parts of the immune system [152, 156]. During infections, phagocytes generate a set of oxidizing agents that have antimicrobial effects but if released into the

extracellular medium, the oxidants can be harmful to the host [156, 157]. The oxidizing agents seem to have an important role in the pathogenesis of certain viral infections, including the common cold and pneumonia [151, 156]. Vitamin C appears to have a protective function in this process. A well studied example of this is the role vitamin C plays in neutrophil function [145]. A diagram of vitamin C transportation into neutrophils is shown in figure 1.7.

Figure 1.7: Ascorbic acid (AA) transportation into neutrophils. In resting cells, ascorbic acid is transported as the reduced form. It is likely that ascorbic acid transport activity is responsible for at least some part of the high internal concentration of ascorbic acid (mmol/L level) in resting cells. Upon neutrophil activation, reactive oxygen species are produced. Extracellular ascorbic acid is oxidized to dehydroascorbic acid (DHA), which is rapidly transported into cells and immediately reduced to ascorbic acid. In this manner, as much as a 10-fold increase in intracellular ascorbic acid concentration can be achieved. The results imply that dehydroascorbic acid formation and transport could occur during inflammation. AA – ascorbic acid, DHA – dehydroascorbic acid.



There are several possible reasons that this ascorbic acid recycling occurs. In neutrophils, ascorbic acid recycling provides rapid increases in intracellular ascorbic acid at the same time that oxidants are produced. Oxidants in neutrophils are produced in the phagosome and on the external surface of the cell membrane, and also leak into the cytosol and extracellular space [145, 158]. Higher concentrations of ascorbic acid in neutrophils could provide better protection against intracellular reactive oxygen intermediates. In neutrophils, these intermediates are an integral component of microbiocidal pathways and mediate apoptosis [159-161]. Increased intracellular ascorbic acid concentrations could be effective in quenching cytosolic and extracellular oxidants and possibly delay neutrophil apoptosis [162, 163]. Prolongation of neutrophil survival could also translate into enhanced microbiocidal activity.

It is also possible that increased intracellular ascorbic acid has an extracellular protective function. This could occur by active extrusion of ascorbic acid under certain conditions or by leakage of intracellular ascorbic acid as neutrophils die. Because ascorbic acid recycling causes such large increases in intracellular ascorbic acid, it could affect extracellular concentrations as a function of neutrophil cell density and direct extracellular diffusion of ascorbic acid into areas of inflammation. Increased extracellular ascorbic acid would be protective against oxidant damage to collagen and surrounding cells.

Vitamin C is essential for neutrophil apoptosis [164]. With vitamin C deficiency, apoptosis is impaired and the neutrophil will undergo necrosis [164]. The effect of vitamin C on neutrophil function may contribute to its effect on the immune system upon infection. Other immune cells, such as monocytes/macrophages, are likely to accumulate high levels of intracellular ascorbic acid by a similar ascorbic acid recycling mechanism.

Vitamin C deficiency also affects macrophage function. Macrophages isolated from vitamin C-deficient guinea pigs demonstrated a significant reduction in the migration ability compared with macrophages from vitamin C-sufficient guinea pigs. Addition of vitamin C to the cultures partially reversed this reduced migration [165]. Vitamin C also inhibits granulocyte macrophage-colony-stimulating factor (GM-CSF)-induced signaling pathways [166]. GM-CSF induces an increase in ROS and uses ROS for signaling functions. Vitamin C may play an important role in interacting with cell signaling pathways that use ROS as secondary messenger molecules [166].

There are few reports on vitamin C's effect on NK cell activity and the existing results are controversial. No effect on NK cell activity by vitamin C was seen in one study using three different strains of mice [167]. However, vitamin C was found to inhibit human NK cell activity in vitro in a dose dependent manner [168]. Moreover, after supplementation of a high dose of vitamin C, NK cell activity increased in patients exposed to toxic chemicals [169].

In vitro studies have given controversial results on the effect of vitamin C on NF-κB activation. This may be because of the existence of transition metal ions in the tissue culture medium or the poor transportation of reduced form of ascorbic acid into the cell. By using dehyroascorbic acid (DHA), which is transported more efficiently than ascorbic acid, mmol/L intracellular concentration of ascorbic acid is achieved and it suppresses TNF-α-induced NF-κB activation by inhibiting IκBα phosphorylation [170], suggesting that vitamin C may play a important role in modulating inflammation and apoptosis. Hence, besides its direct antioxidant function, vitamin C also protects tissue from damage during acute inflammatory responses by inhibiting NF-κB mediated signaling pathways.

4. Gulonolactone oxidase gene knockout mice (*gulo*-/-) can not synthesize vitamin C

The ability of mice and rats to synthesize vitamin C makes it impossible to study the effects of vitamin C deficiency in these animals and puts limitations to the interpretation of vitamin C supplementation studies due to uncontrolled *de novo* vitamin C synthesis.

To create a mouse model of vitamin C deficiency, the *gulo* gene from the mouse was isolated using the known rat sequence and inactivated by gene targeting [171]. *Gulo-/-* mice are like humans, unable to synthesize vitamin C and require vitamin C supplementation. Vitamin C-deficient *gulo-/-* mice show weight loss, reduced plasma antioxidant capacity, increased plasma cholesterol, and blood vessel abnormalities [171].

F. Other antioxidant nutrients overview

1. Vitamin E

Vitamin E is synthesized in plants and has eight isoforms: α-, β-, γ-, and δ-tocopherol; and α-, β-, γ-, and δ-tocotrienol [172]. Most of the vitamin E studies have focused on α-tocopherol, which is the most biologically active form [173]. Although the dietary content of γ-tocopherol is higher than α-tocopherol, α-tocopherol is the major form of vitamin E in the plasma [174]. After being taken up by intestinal cells, vitamin E is released into the circulation in chylomicrons and taken up by the liver with chylomicron remnants. In the liver, vitamin E is incorporated into very low density lipoproteins (VLDL) and excreted back into the circulation. As the VLDL are broken down by lipoprotein lipase, low density lipoproteins (LDL) are formed and from these lipoproteins the vitamin E is transferred to high density lipoproteins (HDL). All of the lipoproteins, chylomicron, VLDL, LDL and HDL have the ability to transfer vitamin E to tissue [175, 176].

The primary function of vitamin E in the body is as a powerful lipid soluble antioxidant. Vitamin E is able to extinguish single oxygen species as well as to terminate the free radical chain-reaction of lipid peroxidation. α-tocopherol acts as an antioxidant either by donating a hydrogen radical to remove the free lipid radical, reacting with it to form nonradical products, or simply trapping the lipid radical [177]. The classical antioxidant actions of vitamin E as a free radical chain reaction breaker are shown in figure 1.8.

Figure 1.8: (A) The radical chain mechanism of peroxidation of lipid-containing bisallylic hydrogens (LH). The process is initiated by the abstraction of a hydrogen on the lipid moiety to produce a carbon-centered lipid radical (L•), which rapidly reacts with oxygen to form a lipid peroxyl radical (LOO•). Then LOO• reacts with another LH to produce LOOH and L•. In the absence of chain-breaking antioxidants, the chain reaction terminates by the reaction of two free radicals.
(B) α-tocopherol (α-TOH) acts as a chain-breaking antioxidant by donating its phenolic hydrogen to LOO• and replacing the latter with the less reactive α-tocopheroxyl radical (α-TO•). α-TOH may also react directly with the initiating radical to prevent LOO• formation in the first place. α-TO• reacts with LOO• forming nonradical products.

A.

Initiation radical oxidant + LH → inactive oxidant + L•

Propagation L• + O → LOO•

Propagation LOO• + LH → LOOH + L•

Termination LOO• + LOO•/L• → nonradical products

B.

Inhibition α-TOH + LOO• → α-TO• + LOOH

Inhibition α-TOH + radical oxidant → inactive oxidant + α-TO•

Termination α-TO• + LOO• → nonradical products

Modified from Upston, JM et al, *The FASEB Journal* (1999);13:977-994.

Vitamin E exerts a preventive effect against cardiovascular diseases by protecting LDL from oxidation. Oxidation of LDL initiates a plaque-forming cascade, which involves the ingestion of oxidized LDL by macrophages forming foam cells. These foam cells secrete proinflammatory chemotactic molecules that attract immune cells which damage endothelium and promote procoagulant activity [178]. The preventive effect of vitamin E on LDL oxidation has been demonstrated in laboratory animals *in vivo* [179], in isolated tissue *ex vivo* [180], and in human populations [178].

An adequate vitamin E intake is important for the normal function of the immune system. In vitamin E deficiency most of the immune parameters show a downward trend, which is associated with increased infectious diseases and the incidence of tumors. In contrast, vitamin E supplementation has various beneficial effects [181]. Vitamin E supplementation changes gene expression profile of T cells and improves T cell function [182]. Vitamin E supplementation has also been shown to improve immune functions in the aged including delayed-type hypersensitivity skin response and antibody production in response to vaccination [183].

2. β-carotene and other carotenoids

β-carotene is also known as provitamin A, because it is one of the most important vitamin A precursors in human diet. The conversion of β-carotene to vitamin A occurs in the small intestine (intestinal mucosa) and is catalyzed by β-carotene dioxygenase [184]. The retinol form is stored in the liver as retinyl esters.

β-carotene is one of a group of naturally occurring compounds called carotenoids. More than 600 carotenoids have been identified and approximately 50 of them have vitamin A activity [185]. β-carotene and other carotenoids have been considered free radical

scavengers [186, 187] and have been shown to have preventive effects against various types of cancer in humans [187].

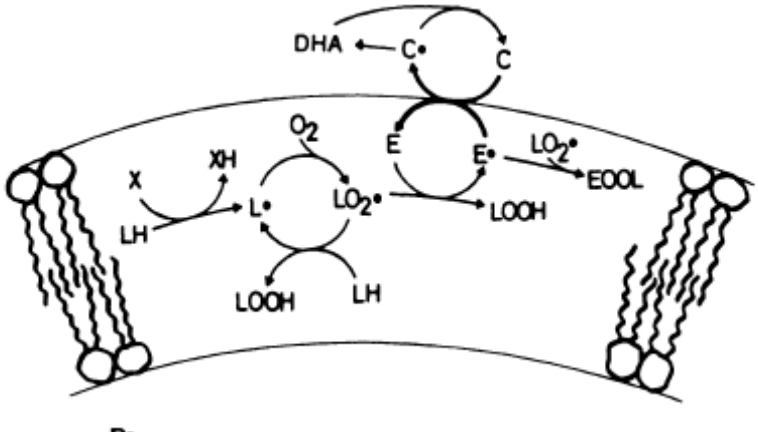
The cancer preventive function of carotenoids may be associated with their effect on the immune system as antioxidants [187, 188]. β-carotene enhances immune cell function. For example, β-carotene supplementation increases the numbers of T lymphocytes and CD4+ T-helper cells in humans [189, 190]. β-carotene supplementation also increases expression on blood monocytes of several receptors required for antigen presenting function [191].

3. Antioxidants function in a collaborative manner

As discussed earlier, vitamin C is a strong antioxidant. However, as a water-soluble vitamin, it is located in the aqueous phase and can not directly scavenge lipophilic radicals within the lipid region of membranes and lipoproteins. However, vitamin C is able to act synergistically with vitamin E by recycling oxidized vitamin E [192-195]. This process is shown in figure 1.9.

Figure 1.9: The protective effect of vitamin E depends on vitamin C to recycle oxidized vitamin E. Tocopherol (E) is regerated from tocopheroxyl radical (E•) by ascorbic acid (C). The chain reaction of lipid peroxidation is also shown.

E – tocopherol, E• - tocopheroxyl radical, C – ascorbic acid, C• - ascorbyl radical, DHA – dehydroascorbic acid, X – chain reaction trigger, LH – lipid, L• - lipid radical, LO_2• - lipid peroxyl radical.

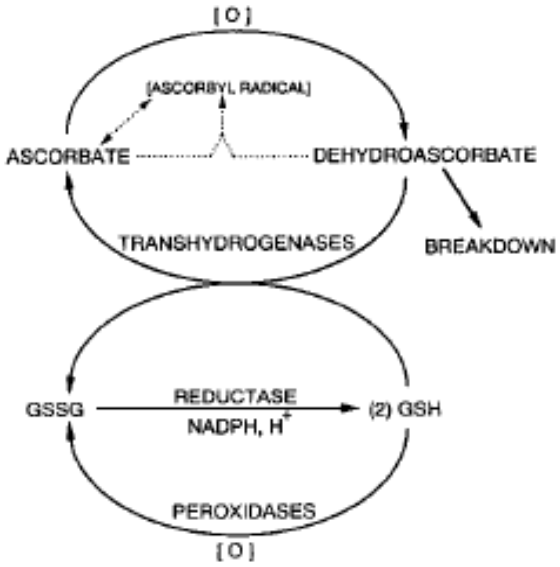


Modified from Niki, E, The American Journal of Clinical Nutrition (1991), 54, 1119S-24S

Vitamin C and glutathione share similar antioxidant function and can spare each other under certain oxidative stressed conditions. L-buthionine-(SR)-sulfoximine (BSO)-induced multiple-organ damage and mortality in GSH-deficient guinea pigs and newborn rats could be prevented by administration of ascorbic acid (but not dehydroascorbic acid) [196]. GSH deficiency is accompanied by a significant decrease in tissue ascorbic acid, suggesting ascorbic acid is expended at a higher rate to compensate for GSH deficiency [196, 197]. In contrast, adult mice are less affected by GSH deficiency compared with guinea pigs and new born rats, due to the ability of mice to synthesize ascorbic acid [196, 198]. On the other hand,

treating ascorbic acid-deficient guinea pigs with GSH delays the onset of scurvy [199]. The interrelationships of GSH and ascorbic acid are shown in figure 1.10.

Figure 1.10: GSH-ascorbic acid interrelationships. Both GSH and ascorbic acid can react with ROS ([O]). GSH can reduce dehydroascorbic acid, regenerating ascorbic acid.



Modified from Meister, A, The Journal of Biological Chemistry (1994), 269 (13), 9397-9400.

There are also interactions between vitamin E and the GSH system. Vitamin E supplementation increases GSH levels in red blood cells in humans [189]. Similar with vitamin C, GSH can also recycle oxidized vitamin E and protect lipids from peroxidation [190].

In summary, antioxidants do not act in isolation, but rather as an intricate network. The interactions between antioxidants play a pivotal role in defending the body from oxidative damages.

G. Oxidative stress may alter the infecting pathogen

It has been known for many years that oxidative stress caused by poor nutrition can affect the immune response to infection. The increase in susceptibility is thought to be the result of an impaired host immune response due to a deficient diet. Although that is the case in many circumstances, the research conducted in our group demonstrated that oxidative stress could increase the severity of infectious diseases by changing the pathogen itself [200]. Dietary deficiencies that lead to oxidative stress in the host can alter a virus genome such that a normally benign or mildly pathogenic virus becomes highly virulent in the deficient, oxidatively stressed host. Once the virus mutations occur, even hosts with normal nutriture can be affected by the newly pathogenic strain [201]. Se deficient mice infected with an amyocarditc strain of coxsakievirus B3 (CVB3/0) developed myocarditis while Se adequate mice did not. Six nucleotide changes were found between the original CVB3/0 strain and the virus isolated from Se-deficient mice while no changes were found in virus that was isolated from Se-adequate mice. Similar results were also seen in an experiment with GPX-1 knockout mice [202].

Since GPX-1 is one of the most important antioxidant enzymes *in vivo*, it is most likely that oxidative stress is the most direct cause of viral genome changes. Published data of vitamin E deficient mice supported this hypothesis [203]. Viruses isolated from vitamin E deficient mice share the six identical nucleotide changes with viruses isolated from Se-deficient mice [203]. Since vitamin E has long been considered an antioxidant vitamin, the fact that Se-deficient, vitamin E-deficient and GPX-1 -/- mice introduced similar changes into virus genome places significant limitations on the mechanistic interpretation of these results. Because both deficiencies led to more or less similar outcomes, it is difficult to

propose biochemical mechanisms involving particular selenoproteins or specific membrane effects of vitamin E. Rather, a broader explanation must be sought, such as the general deleterious impact of oxidative stress on cellular metabolism [200].

To determine whether Se deficiency could have similar effect on a viral family other than enteroviruses, our group used influenza virus for a similar experiment with those that had been done with coxsakievirus. Mice were fed a diet either deficient or adequate in Se for 4 weeks, then inoculated intranasally with influenza A/Bangkok/79/1 (H3N2), a strain that induces mild pneumonitis in normal mice. At all time points post-infection, Se-deficient mice had much more severe pathology then Se-adequate mice [204]. Similar to the experiment conducted with coxsakievirus, when influenza virus isolated from these Se-deficient mice were used to inoculated normal Se-adequate mice, Se-adequate mice developed severe pneumonitis, suggesting the influenza virus genome was altered. Sequencing data confirmed that the M gene, which encodes the virus matrix proteins, was markedly altered when compared with the isolates from Se-adequate mice [204].

The possible mechanisms for these virus genome changes are currently under investigation, among which two are most likely. **1) Direct oxidative damage to viral RNA by reactive oxygen species.** That is, when replicating in an oxidatively stressed host, the virus genome is susceptible to the damage caused by the reactive oxygen species in the host cells. This damage introduces random mutations into the viral genome. This is especially true for RNA viruses like coxsakievirus and influenza virus, which use an RNA polymerase without proof-reading functions. At the same time, replication of the virus will favorably select those mutations that give the virus more virulence. After many cycles of replication, the most virulent mutant will become dominant. **2) Viral selection via quasispecies.** An

RNA virus is really a collection of closely related mutants ("quasispecies") rather than a single uniform molecular entity [205]. That is, the nucleotide sequence of an RNA viral genome represents a consensus or average base composition derived from a population distribution of viruses [200]. The occurrence of such pre-existing micro-heterogeneous genomic structures would allow the RNA virus to adapt quickly to changes in its environmental conditions [206]. It is possible that an oxidatively stressed host environment would favor the outgrowth of certain quasispeices of an RNA virus, although the mechanism is still not clear. **Finally, other alternate explanations may exist in addition to the two given above and two or more of them could act in a collaborative manner. Recently, Broome et al. demonstrated that increased poliovaccine mutants were shed from Se-deficient human populations [207]. Gay et al (manuscript in press) demonstrated that coxsakievirus mutations occurred in aging mice, which are known to be oxidative stressed.** Our research and that of others demonstrate that host nutritional status is a powerful influence on the in vivo evolution of RNA viral pathogens.

H. Questions arise from earlier observations

The studies with influenza A/1/79 infected Se deficient mice raise the following questions: 1) How will a mouse-adapted virulent strain of influenza, rather than a mild strain, behave in Se-deficient mice? 2) Will the deficiency in other antioxidant nutrients, such as vitamin C, have a similar effect on the immune response to influenza virus infection and viral mutation?

In contrast to the mild influenza A/Bangkok/1/79, influenza A/PR8/34 (PR8) is a mouse-adapted influenza virus which replicates well in the mouse under normal conditions [208], causing a severe inflammatory response [45]. To address the first question, we infected Se-deficient mice with PR8 and measured the immune response in infected mice and screened the viral genome post infection for possible mutations. To address the second question, we used influenza infected vitamin C-deficient *gulo-/-* knockout mice and measured the immune response to influenza virus infection as well as possible viral genome mutations. Taken together with our previous data, the studies in the current dissertation deepened our understanding of the effect of nutritionally-induced oxidative stress on the immune response to viral infection, as well as the distinctive immunomodulatory properties of individual nutrients.

Chapter II

Selenium deficiency induced an altered immune response and increased survival following influenza A/PR8/34 infection

Wei Li, Melinda A. Beck

Submitted to *Experimental Biology and Medicine*

A. ABSTRACT

This study was designed to determine the effect of selenium deficiency on the immune response to infection with a virulent strain of influenza virus, influenza A/Puerto Rico/8/34. Previous work in our laboratory demonstrated that Se-deficient mice infected with a mild strain of influenza virus, influenza A/Bangkok/1/79, developed much more severe lung pathology compared with Se-adequate mice. Immune function was altered in the Se-deficient mice. The viral genome changed to a more virulent genotype. In this study, we tested whether Se deficiency would have a similar effect on mice infected with a more virulent, mouse-adapted strain of influenza virus. Three-week old male mice were fed Se-adequate or Se-deficient diet for 4 weeks prior to inoculation with influenza A/PR8/34. There was no difference in lung influenza viral titer between Se-deficient and Se-adequate mice. Se-deficient mice had less MIP-1α and RANTES production at the transcriptional and protein level in the lung post infection. Se-deficient mice also had higher levels of IL-2 expression followed by a higher level of IL-4 expression in the lung. At day 7 post-infection, Se-deficient mice had a lower mortality rate (0%) compared with Se-adequate mice (50%). Sequencing of the virus isolated from infected Se-adequate and Se-deficient mice did not detect viral genome mutations in either group. This study demonstrated that Se-deficient mice had an altered immune response to an infection with a virulent strain of influenza virus. This altered immune response was beneficial for protecting the mice from influenza virus-induced mortality.

B. INTRODUCTION

Selenium (Se) is of fundamental importance to human health. It is an essential component of several major metabolic pathways, including thyroid hormone metabolism and antioxidant enzyme defense systems. Se is incorporated as selenocysteine at the active site of a wide range of selenoproteins. Under physiological conditions the Se in selenocysteine is an extremely efficient biological catalyst. Among the selenoproteins with identified biological functions are the antioxidant enzymes glutathione peroxidase (GPX) and thioredoxin reductase (TrxR).

Dietary Se is essential for a healthy immune system [126] and Se influences both the innate and the adaptive immune responses [124, 125]. The effects of Se deficiency include reduced T-cell numbers and impaired lymphocyte proliferation and function [117]. Se supplementation enhances T cell responses, stimulates antibody production and protects immune cells from oxidative-induced damage. The diverse effects of Se on immune function have been previously reviewed [126].

Infection with influenza virus causes a great deal of morbidity and mortality worldwide each year. In the U.S. alone, influenza virus infection results in over 36,000 deaths and 114,000 hospitalizations per year [13]. Infection with influenza virus causes damage to both the lungs and airways due to inflammatory responses. Although the immune response is critical for the recovery from viral infection, it is also responsible for the lung inflammation that contributes significantly to lung pathology.

Previous work in our laboratory demonstrated that mice deficient in Se, which led to a decrease in the Se-containing enzyme glutathione peroxidase (GPX), were much more susceptible to infection with a mild influenza virus. Specifically, Se-deficient mice

infected with a non-mouse adapted strain of influenza virus, influenza A/Bangkok/1/79, developed much more severe lung pathology compared with Se-adequate mice [204]. Immune function was altered in the infected Se-deficient mice, and the viral genome had changed in Se-deficient animals to a more virulent genotype. Once these changes occurred, even mice with normal Se status would develop severe pathology when infected with the newly mutated influenza virus [209]. Because influenza A/Bangkok/1/79 is a human influenza virus strain that was not adapted to grow efficiently in mice, we questioned how a mouse-adapted, virulent strain of influenza virus would behave in a Se-deficient animal.

C. MATERIALS AND METHODS

Influenza virus. Influenza A/Puerto Rico/8/34 was propagated in 10-day-old embryonated hen's eggs [210]. The virus-containing allantoic fluid was collected and stored at -80°C. This mouse-adapted strain of influenza virus causes a strong inflammatory response in normal mice [45].

Mice. Three-week old male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were housed 4/cage in the University of North Carolina at Chapel Hill animal facility, which is fully accredited by the American Association of Laboratory Animal Care. All mice were maintained under protocols approved by the Institutional Animal Use and Care Committee of the University of North Carolina at Chapel Hill. For all experiments, mice were provided with Se-adequate or Se-deficient diets for four weeks prior to inoculation with influenza PR8. At baseline and various time points following infection, mice were killed and the tissues were collected for the determination of lung pathology, liver glutathione peroxidase (GPX) activity and glutathione levels, and lung proinflammatory chemokine and cytokine levels.

Diet. The diet was obtained from Harlan (Indianapolis, IN). Se was added to the Se-adequate diet as sodium selenite. The Se content of the experimental diets was determined to be 200 ±8 μg Se/kg for the Se-adequate diet and below the instrumental detection limit of 2.7 μg Se/kg for the Se-deficient diet.

Infection of mice. Mice were lightly anesthetized with an intraperitoneal injection of ketamine (0.022 mg) and xylazine (0.0156 mg). Following anesthesia, mice

were infected intranasally with 2.5 pfu (plaque-forming unit) of influenza A/PR8/34 in 0.05 mL sterile PBS.

Glutathione peroxidase (GPX) activity. Liver GPX activity was determined according to the method of Paglia and Valentine [211]. Briefly, liver was homogenated in 4X volume of Na/K phosphate buffer. Master mix (10 parts 0.4 mol/L Na phosphate buffer, 5 parts NaN3, 4 parts dddH_2O), 5mmol/L GSH and 9 µL of glutathione reductase, and 5 µL liver homogenate were combined. After blanking, 80 µL of 6 mM NADPH was added to each sample. Samples were incubated for 1 min at 37°C followed by addition of 50 µL H_2O_2. Absorption at A340 was immediately measured for 1 min at 20 sec intervals. One mU of enzyme activity was defined as 1 nmol of NADPH oxidized to NADP per mg of protein per min.

GS and GSH analysis. Total glutathione (GS) and reduced glutathione (GSH) were analyzed in tissue extracts prepared in 5% 5-sulfosalicylic acid (S2130, Sigma) using a GR coupled recycling assay [212]. GSH disulfide (GSSG) concentrations were determined in extracts pretreated with 2-vinylpyridine (132292, Aldrich). GSH was determined by subtracting GSSG from total GS.

Histopathology of lungs. The left lung was removed and inflated with 4% paraformaldehyde in 0.1M Na phosphate buffer (pH 7.2). Sections (6 µm) were fixed in acetone and stained with hematoxylin-eosin. The extent of inflammation was graded without knowledge of the experimental variables by two independent investigators. Grading was performed semiquantitatively according to the relative degree (from lung to lung) of inflammatory infiltration. The scoring was as follows: 0, no inflammation; 1+, mild influx of inflammatory cells with inflammatory infiltrates clustered around vessels;

2+, increased inflammation with approximately 25-50% of the total lung involved; 3+, severe inflammation involving 50-70% of the lung; and 4+, almost all lung tissue contains inflammatory infiltrates.

Quantitation of viral titer by real time PCR. To determine lung viral titers, half of the right lung was removed, and total RNA was isolated using the TRIzol method (Invitrogen). Reverse transcription was carried out using Superscript II First Strand Synthesis kit (11904-018, Invitrogen) using random hexamer primers. Expression of the influenza matrix (M1) gene and GAPDH were determined by quantitative real time PCR (qRT-PCR) as described [213]. Fluorescent reporters were detected using Bio-Rad (Hercules, CA) iCycler PCR machine and primers and probes were obtained from Applied Biosystems (Foster City, CA). The levels of mRNA for GAPDH were determined for all samples and were used to normalize expression of the influenza M1 gene. Data were converted to hemagglutination units (HAU) using real time PCR standards made from the virus stock with known HAU titer.

Quantitation of lung chemokine and cytokine mRNA by real time PCR. mRNA levels for murine RANTES, MIP-1α, IL-2, IL-4, and GAPDH were determined using qRT-PCR, as described above. All data were expressed as the ratio to the day 0 (uninfected) levels of the Se adequate group. There were no statistical differences between Se-adequate and Se-deficient mRNA levels at day 0.

Quantitation of lung chemokine protein by ELISA. Half of the right lung was removed and homogenized in 1 mL PBS. ELISA for murine RANTES and MIP-1α were performed with ELISA kits (DY478, DY450, R&D Systems) according to protocols provided by the manufacturer. Results were normalized to the amount of total protein.

Virus sequencing. Viral RNA isolation and reverse transcriptation were carried out as described above. Primers were designed for amplification of the matrix (M) and hemagglutinin (HA) genes. PCR was performed with the following primer sets: M: 5'-GATGAGTCTTCTAACCGAGGT-3' and 3'-AAACAGTCGTATCTCGACCTCA-5'; HA: 5'-TGAAGGCAAACCTACTGGTCC-3' and 3'-ACCTAGAAACGTCACGTCTT-5'. PCR products were purified using the QIA quick PCR purification kit (Qiagen, Valencia, CA). DNA was sequenced at the UNC-CH Automated DNA Sequencing Facility on a Model 377 DNA sequencer (Applied Biosystems Division, Perkin Elmer, Boston, MA) by using the ABI Prism^{TM} Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase (Applied Biosystems Division, Perkin Elmer, Boston, MA). Sequencing data were analyzed with Sequencher 4.5 (Gene Codes Corporation, Ann Arbor, MI).

Statistical analysis. Lung pathology data were analyzed by student t-test. Glutathione peroxidase (GPX) activity and glutathione data were analyzed by two-way ANOVA followed by Tukey HSD test. Real time PCR and ELISA data were analyzed by Kruskal-Wallis test. All statistical analyses were performed with JMP software (SAS, Cary, NC).

D. RESULTS

Se-deficient mice had decreased glutathione peroxidase (GPX) activity. Prior to influenza virus infection (day 0) and at 3 and 7 days post infection, mice fed the Se-deficient diet had significantly lower levels of GPX activity in the liver compared with mice fed the Se-adequate diet, indicating that Se deficiency occurred in the mice on the deficient diet. Infection induced an increase in GPX activity in the Se-adequate mice at day 3 post infection compared with the Se-adequate mice at day 0 (figure 2.1). The increased GPX activity in infected Se-adequate mice suggests that these mice increased the amount of GPX in response to the infection-induced oxidative stress, while Se-deficient mice were not able to do so due to the limited supply of Se, which is essential for the synthesis of GPX.

Se-deficient mice had less amounts of total glutathione (GS) and reduced form of glutathione (GSH). Prior to influenza virus infection (day 0), Se-deficient mice had significantly lower levels of total GS and GSH in the liver compared with Se-adequate mice, suggesting that Se-deficient mice were oxidatively stressed prior to infection. Following infection, GS and GSH levels were significantly decreased at day 7. Infection also induced a decrease in GS and GSH levels in Se-adequate mice at 3 and 7 days post infection, suggesting that influenza infection induced oxidative stress in Se-adequate mice as well (figure 2.2).

Se-deficient mice had a lower influenza-induced mortality rate. At day 7 post influenza virus infection, Se-adequate mice had a 50% mortality rate while no mice in the Se-deficient group died (figure 2.3).

Lung influenza viral titer. Because of the higher mortality rate in the infected Se-adequate mice, we reasoned that lung influenza viral titers would be higher in Se-adequate animals. However, there were no differences in viral titer between Se-adequate and Se-deficient groups. Peak influenza viral titers occurred at day 3 post infection and decreased at day 7 in both Se-adequate and Se-deficient mice (figure 2.4).

Se-deficient mice had lower RANTES and MIP-1α levels in the lung post influenza virus infection. Previous studies have demonstrated the importance of chemokines in the development of influenza-induced inflammation [36, 214, 215]. To determine if Se deficiency influenced chemokine production, both lung mRNA and protein levels for RANTES and MIP-1α and mRNA levels for MCP-1 were measured. Chemokine mRNA levels for MCP-1 increased at 1, 3, and 7 days post infection, but there was no difference in the levels between Se-adequate and Se-deficient mice (data not shown). However, although mRNA levels for RANTES and MIP-1α also increased post infection, Se-adequate mice had a greater response compared with Se-deficient mice (figure 2.5 A and C). To confirm that mRNA levels correlated with protein levels, lung RANTES and MIP-1α protein levels were measured. Se-deficient mice had less amounts of RANTES and MIP-1α proteins at 1 and 3 days post infection (figure 2.5 B and D), correlating with mRNA data.

Se-deficient mice had higher levels of IL-2 expression followed by a higher level of IL-4 expression in the lung post influenza virus infection. Cytokine production in the lungs is also a hallmark characteristic of influenza infection. To determine if Se deficiency could affect cytokine release, we measured lung mRNA for levels of TNF-α, IL-1β, IL-12, IL-2, IL-4, IFN-γ and IL-10. Only IL-2 and IL-4 levels

demonstrated significant differences between Se-adequate and Se-deficient mice (figure 2.6 A and B). IL-2 mRNA levels were higher in Se-deficient mice at 1 and 3 days post infection (6A) and IL-4 mRNA level was higher at day 7 post infection in the Se-deficient mice compared with the Se-adequate controls (6B).

No mutations were detected in influenza A/PR8/34 viral genome after replicating in Se-adequate or Se-deficient mice. The matrix (M) gene and (hemagglutinin) HA gene of the influenza PR8 viral genome isolated from Se-adequate and Se-deficient mice at day 7 post infection were sequenced and compared with the sequence of the virus stock used for infection. No mutations were detected.

E. DISCUSSION

The fundamental importance of Se to optimal immune function has been widely demonstrated. An adequate Se intake is essential for an appropriate immune response to various infectious diseases. For example, Se supplementation results in more rapid poliovirus clearance [207] as well as lowered hospitalization rates among HIV infected patients [216]. Se deficiency is associated with Keshan disease, a disease correlated with coxackievirus infection of the heart muscle [217]. By using a murine model of coxsackievirus B3 (CVB3)-induced myocarditis, our laboratory demonstrated that Se-deficient mice were more susceptible to the cardiopathologic effects of the virus. In addition, a normally benign strain of CVB3 becomes virulent in Se-deficient mice [218]. Previous work in our laboratory also demonstrated that Se-deficient mice were more susceptible to infection with mild influenza virus. Specifically, Se-deficient mice infected with a mild strain of influenza virus, influenza A/Bangkok/1/79, developed much more severe lung pathology compared with Se-adequate mice. Immune function was altered in the infected Se-deficient mice, and the viral genome had changed in the deficient animals to a more virulent genotype [204, 209].

The strain of influenza virus used in these previous studies, influenza A/Bangkok/1/79, is a human strain of influenza virus that induces a mild inflammatory response in normal mice. In this study, a mouse-adapted strain of influenza virus, influenza PR8 was used. This strain of virus induces a much more severe lung pathology compared with the non-mouse adapted Bangkok strain. Both Se-adequate and Se-deficient mice had severe lung inflammation following infection with PR8. Surprisingly, PR8 infected Se-deficient mice demonstrated no mortality at day 7 compared with Se-

adequate mice which had a 50% mortality rate. This was in contrast to Se-deficient Bangkok-infected mice. In order to determine the reason for the lower mortality and lung pathology in the Se-deficient mice, we measured lung viral titers. Less lung pathology may have been related to lower lung viral titers in the Se-deficient mice. However, no differences in the lung influenza viral titers were found between Se-adequate and Se-deficient mice, suggesting that Se levels did not influence the ability of the host to eradicate the virus.

Another possibility for the difference in lung pathology may be in the production of lung chemokines and cytokines, which are responsible for generating the lung inflammation post infection. We found that influenza infected Se-deficient mice had less production of the chemokines RANTES and MIP-1α in the lungs compared with Se-adequate mice. Chemokines are potent chemoattractant cytokines and have been considered the main candidate molecules responsible for the selective recruitment of distinct leukocyte populations. RANTES is produced by CD8+ T cells, epithelial cells, fibroblasts and platelets and plays a key role in the immune response to viral infection [26]. MIP-1α is produced by a variety of cell types, including monocytes, macrophages, mast cells, Langerhans cells, fibroblasts, and T cells. MIP-1α is primarily chemotactic for B cells, activated CD8+ T cells, natural killer (NK) cells, and eosinophils [30-33]. MIP-1α also increases cell adhesion by inducing ICAM-1 expression [35].

Influenza virus infected MIP-1α knockout mice have significantly less lung inflammation compared with MIP-1α wild-type controls, suggesting that MIP-1α plays a critical role in the inflammatory response to influenza virus infection [36]. Because chemokines are important mediators of inflammation, the lower levels of RANTES and

MIP-1α in the lungs of the Se-deficient mice may have prevented the severe lung inflammation that caused mortality in the Se-adequate mice.

Infection with influenza virus induces a strong Th1 type response, resulting in expansion of influenza-specific CD8+ T cells, which play a significant role in viral clearance. However, the Se-deficient mice had a more Th2-like pattern of cytokine expression. IL-4 is an important mediator of Th2 type responses and the IL-4 expression was much higher in the lungs of the Se-deficient mice at day 7 post infection. IL-2 mRNA levels were also higher in the lungs of the Se-deficient mice at 1 and 3 days post infection compared with the Se-adequate controls. IL-2 stimulates T cell activation and expansion [219, 220], especially the development of Th2 cells by stabilizing the accessibility of the IL-4 gene [221]. *In vivo*, IL-2 neutralization inhibits IL-4 production [221]. Thus, the higher mRNA level of IL-4 at day 7 may have been stimulated by the earlier higher levels of IL-2 in Se-deficient mice. In addition, RANTES and MIP-1α have been correlated with a Th1 type response [59-62] and MIP-1α drives the development of Th1 cells *in vitro* [222], again suggesting that the lower levels of these chemokines in the Se-deficient mice skewed the response towards a Th2 rather than a Th1 response.

Why does Se deficiency favor a Th2 response? The lower GSH levels in Se-deficient mice may be a possible explanation. GSH levels in the immune cells play a pivotal role in determining the Th1/Th2 balance. GSH depletion in antigen presenting cells (APCs) shifts the immune response toward a Th2 response both *in vitro* and *in vivo* [223]. The intracellular GSH levels have also been correlated with the Th1 type cytokine production versus the Th2 type cytokine production by macrophages and CD4+ T cells [224]. The effect of GSH levels on Th1/Th2 balance is partly mediated by IL-12 and IL-4

[223, 224]. Our data complement these studies by showing a higher level of IL-4 expression in Se-deficient mice. Although a difference in IL-12 expression between Se-adequate and Se-deficient mice was not observed in our study, the diminished Th1 response in Se-deficient mice may be explained by the changes in production of other chemokines and cytokines by APCs and macrophages, such as RANTES, MIP-1 and possibly IL-18.

In addition, effects of Se deficiency on immune function may not be solely explained by the change in GSH levels. Approximately 25 selenoproteins have been identified, many with unknown biological function [78]. It is possible some of these selenoproteins have immunomodulatory functions. With the progression of our understanding of the function of selenoproteins, more detailed mechanisms will be elucidated.

Previous studies in our laboratory had demonstrated that influenza A/Bangkok/1/79, a mild strain of influenza virus in mice, underwent substantial genetic mutations in viral matrix (M) gene after replicating in Se-deficient mice, which turned the virus into a more virulent strain [209]. However, in the current study with influenza PR8 infection, no mutations were detected in this gene. We also sequenced the viral hemagglutinin (HA) gene, a viral RNA segment with a high mutation rate, and found no mutations. As a human strain of influenza virus, influenza A/Bangkok/1/79 does not replicate efficiently in mice under normal conditions. In contrast, influenza PR8 is a mouse-adapted strain of influenza, and replicates well in mice under normal conditions. Thus, less host-adapted viruses may be more susceptible to mutations induced by a nutritional deficiency.

The lower mortality rate in Se-deficient mice was not caused by a lower viral titer, as Se-adequate mice and Se-deficient mice had similar viral titers in the lung. Instead, the lower mortality was a result of the weakened inflammatory response in these mice. Although the weakened immune response increased the survival of the Se-deficient mice, this may not be beneficial for an infection in which the inflammatory response plays a more critical role in viral eradication. Thus, a careful balance between inflammation that is required for virus control vs. inflammation that damages tissues must be struck. In this study, Se deficiency tipped the balance in favor of reduced inflammation, which proved to be beneficial to the animals.

In summary, Se-deficient mice had an altered immune response to influenza virus infection, which was characterized by a diminished Th1 type response and an enhanced Th2 type response. Less production of chemokines in the lungs of Se-deficient mice contributed to less lung pathology and the higher survival rate. No genome changes occurred in the virus. Further studies to investigate the influence of Se on immune function are warranted.

Figure 2.1: Glutathione peroxidase (GPX) activity in the liver prior to influenza virus infection (day 0), and at 3 and 7 days post infection. Mice were fed the Se-adequate or Se-deficient diets for 4 weeks prior to infection. Data are expressed as mean ± SEM. n = 5. Asterisks indicate significant difference between the Se-adequate and Se-deficient groups (P < 0.0001). § indicate significant difference between the level post-infection and the level prior to infection in either the Se-adequate group or Se-deficient group (P < 0.05).

Figure 2.2: Total glutathione (GS) and reduced glutathione (GSH) in the liver prior to influenza virus infection (day 0) and at 1, 3, and 7 days post infection. Mice were fed the Se-adequate or Se-deficient diets for 4 weeks prior to infection. Data are expressed as mean ± SEM. n = 8. Asterisks indicate significant difference between Se-adequate and Se-deficient groups (P < 0.01). § indicate significant difference between the level post-infection and the level prior to infection in either the Se-adequate group or Se-deficient group (P < 0.01).

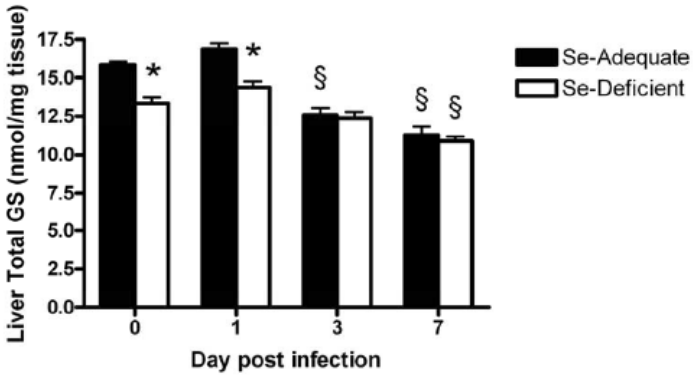


Figure 2.3: Survival rate post influenza virus infection in Se-adequate and Se-deficient mice. Mice were fed the Se-adequate or Se-deficient diets for 4 weeks prior to infection. Data are expressed as percentage of survival. n = 8.

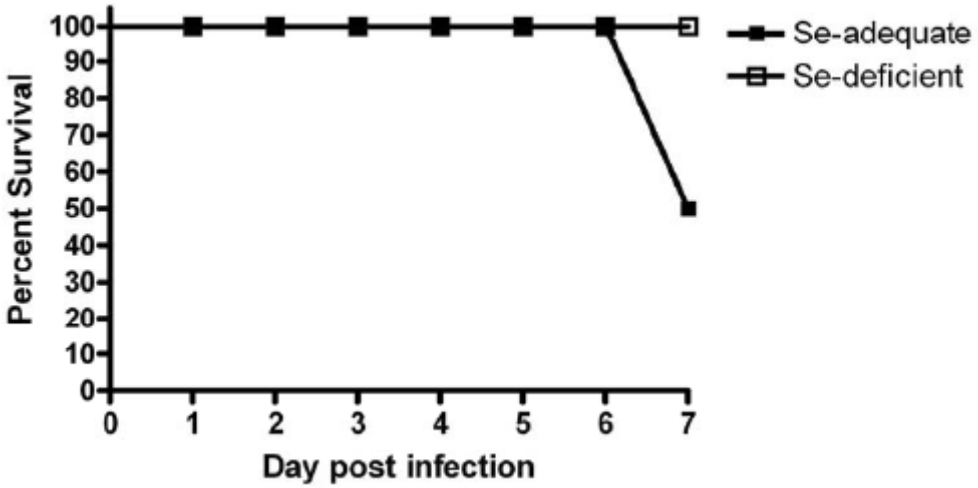


Figure 2.4: Lung influenza viral titers prior to influenza virus infection (day 0) and at 1, 3, and 7 days post infection. Mice were fed the Se-adequate or Se-deficient diets for 4 weeks prior to infection. Data are expressed as mean ± SEM. n = 8.

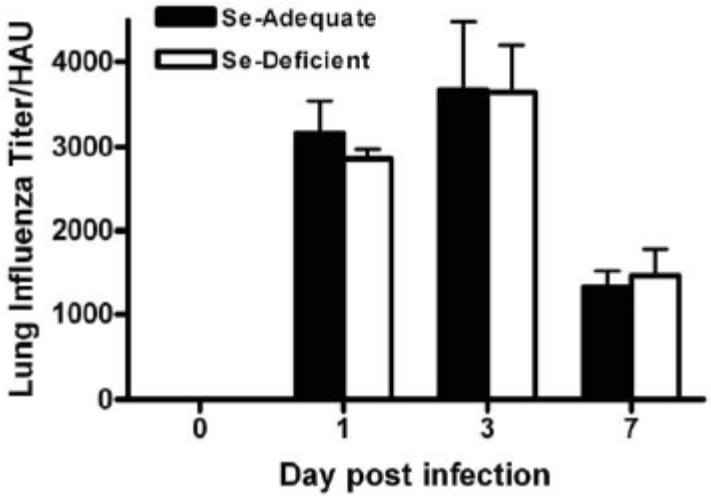


Figure 2.5: Lung mRNA and protein levels for RANTES and MIP-1α from Se-adequate or Se-deficient mice prior to influenza virus infection (day 0) and at 1, 3, and 7 days post infection. mRNA data are expressed as the ratio to the day 0 levels of the Se-adequate group ± SEM. Protein data are expressed as ng RANTES or MIP-1α/mg total tissue protein ± SEM. n = 8. Asterisks indicate significant difference between Se-adequate and Se-deficient groups (P < 0.05).

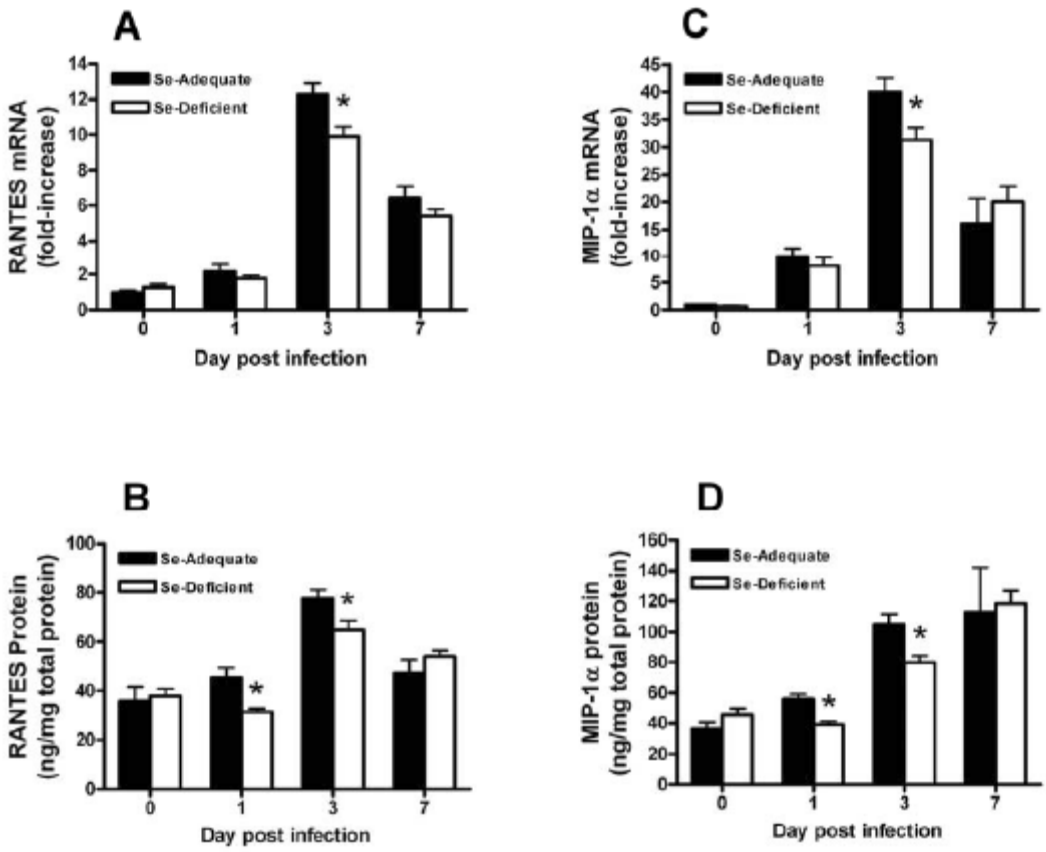
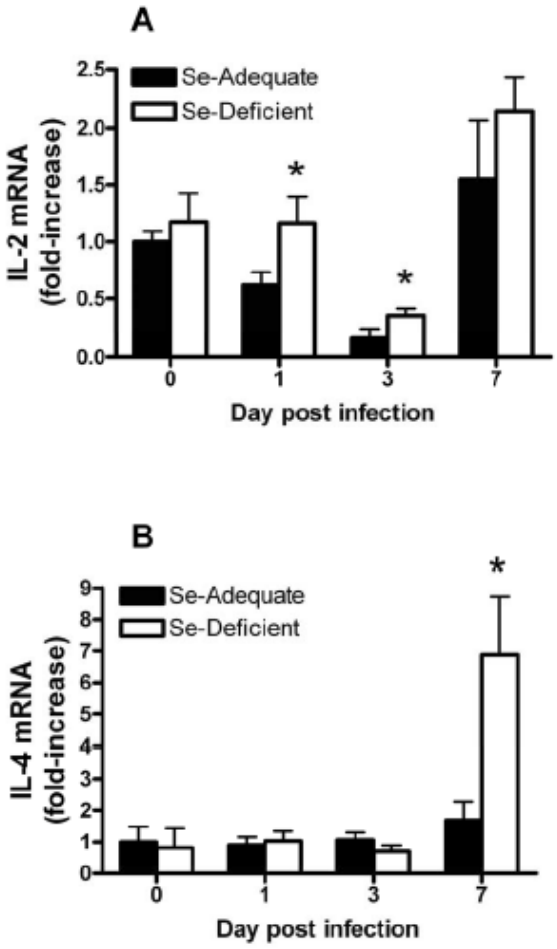


Figure 2.6: Lung mRNA levels for IL-2 and IL-4 from Se-adequate or Se-deficient mice prior to influenza virus infection (day 0) and at 1, 3, and 7 days post infection. Data are expressed as the ratio to the day 0 levels of the Se-adequate group ± SEM. n = 8. Asterisks indicate significant difference between Se-adequate and Se-deficient groups (P <0.05).



Chapter III

Vitamin C deficiency increases the lung pathology of influenza virus infected *gulo-/-* mice

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A. ABSTRACT

This study was designed to determine the effects of vitamin C deficiency on the immune response to infection with influenza virus. L-gulono-γ-lactone oxidase gene inactivated mice (*gulo -/-* mice) require vitamin C supplementation for survival. Five-week old male and female *gulo-/-* mice were provided with water or water containing 330 mg/L vitamin C for three weeks prior to inoculation with influenza A/Bangkok/1/79. There were no differences in lung influenza virus titers between vitamin C adequate and deficient mice; however, lung pathology in the vitamin C deficient mice was greater at 1 and 3 days post infection, but less at day 7 compared with vitamin C adequate mice. There was a clear sex difference of the effect of vitamin C deficiency on the immune response: Male vitamin C deficient mice had a greater expression of mRNA for RANTES, IL-1β and TNF-α in the lungs at day 1 post infection compared with male controls. However, at day 3 post infection, male vitamin C deficient mice had a less expression of mRNA for RANTES, MCP-1 and IL-12 compared with male controls. None of these differences were observed in female mice. Vitamin C deficient male mice also had a greater NF-κB activation as early as 1 day post infection compared with male controls. These data suggest that vitamin C is required for an adequate immune response in limiting lung pathology post influenza virus infection.

B. INTRODUCTION

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) play a pivotal role in the regulation of the immune response to infection. As an antioxidant vitamin, the effect of vitamin C on the immune response has been widely studied. Vitamin C is accumulated in mmol/L concentration in neutrophils, lymphocytes, monocytes, and platelets [141, 145-150], suggesting that it may be important for the functioning of the immune system. Although the mechanisms whereby vitamin C affects the immune system are poorly understood, there are studies that suggest that phagocyte function, T cell proliferation and production of inflammatory cytokines are affected by vitamin C status [151-155]. During infection, activated phagocytes produce oxidizing agents that have antimicrobial effects but if released into the extracellular medium, can be harmful to the host [156, 157]. To neutralize the effects of the increased presence of oxygen radicals, the cells utilize a variety of antioxidative mechanisms including antioxidant enzymes and antioxidant nutrients such as vitamin C.

Although studies of the effect of vitamin C on colds have been reported [225-228], there are few data on the effect of vitamin C on influenza virus infection. Infection with influenza virus causes a great deal of morbidity and mortality worldwide each year. In the U.S. alone, influenza virus infection results in over 36,000 deaths per year. Infection with influenza virus causes damage to both the lungs and airways due predominantly to inflammatory responses. Although the immune response is critical to the recovery from influenza infection, it is also responsible for the lung inflammation that contributes significantly to lung pathology. Administration of vitamin C alone or vitamin C in combination with vitamin E reduces lipid peroxidation and monooxygenase enzyme

activation induced by influenza virus infection [229], which may have an effect on lung pathology post infection.

Vitamin C is synthesized from glucose in the liver of most mammalian species, except for humans, non-human primates, guinea pigs and some fruit bats. These species lack the enzyme L-gulono-γ-lactone oxidase, which is essential for synthesis of the vitamin C immediate precursor 2-keto-1-gulonolactone. The DNA encoding gulonolactone oxidase in humans has undergone substantial mutation, resulting in the absence of a functional enzyme [132, 133]. The ability of mice and rats to synthesize vitamin C makes it impossible to study the effects of vitamin C deficiency in these animals and puts limitations to the interpretation of vitamin C supplementation studies due to uncontrolled *de novo* vitamin C synthesis. However, the creation of *gulo-/-* mice [171] provides an excellent model for studying vitamin C deficiency and its effect on the immune response to viral infection.

Previous work in our laboratory has demonstrated the importance of selenium (as an antioxidant) and vitamin E in optimizing the immune response to influenza virus infection [201, 230, 231]. To determine if vitamin C was also important for immune function, we investigated the effects of vitamin C deficiency on the infection by a mild strain of influenza virus (influenza A Bangkok/1/79) in *gulo -/-* mice

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C. MATERIALS AND METHODS

Influenza virus. Influenza A/Bangkok/1/79 was propagated in 10 day old embryonated hen's eggs [210]. The virus-containing allantoic fluid was collected and stored at -80°C. This human strain of virus causes a mild inflammatory response in normal mice.

Mice. *Gulo-/-* mice with a mixed genetic background (129 and B6) were bred in the University of North Carolina at Chapel Hill animal facility, which is fully accredited by the American Association of Laboratory Animal Care. Mice were housed 3-4/cage. All mice were maintained under protocols approved by the Institutional Animal Use and Care Committee of the University of North Carolina at Chapel Hill. For experiments, five-week old male and female g*ulo-/-* mice (4-5 mice/group) were provided with water or water containing 330 mg/L vitamin C for three weeks prior to inoculation with influenza A/Bangkok/1/79. At baseline prior to infection (day 0) and 1, 3 and 7 days post-infection, mice were killed by cervical dislocation and the tissues were collected for the determination of lung pathology, tissue ascorbic acid (the reduced form of vitamin C), glutathione levels, the expression of pro-inflammatory chemokines and cytokines, and NF-κB activation in the lung.

Infection of mice. Mice were lightly anethetized with an intraperitoneal injection of ketamine (0.022 mg) and xylazine (0.0156 mg). Following anesthesia, mice were infected intranasally with 10 HAU (Hemagglutinating Unit) of influenza A/Bangkok/1/79 in 0.05 mL sterile PBS.

Tissue ascorbic acid measurement. Ascorbic acid (the reduced form of vitamin C) was measured by the α,α'-dipyridyl method as described [232].

GSH anlaysis. GSH and GSH-disulfide (GSSG) were analyzed in lung tissue extracts prepared in 5% 5-sulfosalicylic acid (S2130, Sigma) using a glutathione reductase-coupled recycling assay [212]. GSSG concentrations were determined in extracts pre-treated with 2-vinylpyridine (132292, Aldrich).

Histopathology of lungs. The left lung was removed and inflated with 4% paraformaldehyde fixative in 0.1M Na phosphate buffer (pH 7.2). Sections (6 μm) were fixed in acetone and stained with hematoxylin-eosin. The extent of inflammation was graded without knowledge of the experimental variables by two independent investigators. Grading was performed semiquantitatively according to the relative degree (from lung to lung) of inflammatory infiltration. The scoring was as follows: 0, no inflammation; 1+, mild influx of inflammatory cells with inflammatory infiltrates clustered around vessels; 2+, increased inflammation with approximately 25-50% of the total lung involved; 3+, severe inflammation involving 50-70% of the lung; and 4+, almost all lung tissue contains inflammatory infiltrates.

Western blot. Cytosol and nuclear protein extracts were prepared from lung tissue using Nuclear/Cytosol Fractionation Kit (K266-100, Biovision, Mountain View, CA) following protocols provided by the manufacturer. Extracts were separated by 12% (wt/vol) sodium dodecyl sulfate (SDS) polyacrylamide gels and electrophoretically blotted onto Immobilon-P membranes (Millipore) according to standard procedures. The membranes were blocked with 5% non-fat milk and treated with rabbit anti-mouse IκBα (9242, Cell Signaling Technology, Danvers, MA) and anti-mouse β-actin (4967, Cell Signaling Technology, Danvers, MA) overnight at 4°C. Membranes were then incubated with a secondary goat anti-rabbit IgG conjugated with horseradish peroxidase (7074, Cell Signaling Technology, Danvers, MA) for 1h at room temperature and stained with SuperSignal West Pico

Chemiluminescent Substrate (Pierce, Rockford, IL). The chemiluminescent signals were acquired using a 16-bit CCD camera (GeneGnome system; Syngene, Frederick, MD). Densitometry analysis was conduct using the GeneSnap software (Syngene).

Electrophoretic mobility shift assay (EMSA). EMSA was performed with the Panomics' EMSA Kit (AY1030, Panomics, Redwood City, CA) according to protocols provided by the manufacturer.

Quantitation of viral titers. Half of the lung was removed, and total RNA was isolated using the TRIzol method (Invitrogen). Reverse transcription was carried out using Superscript II First Strand Synthesis kit (11904-018, Invitrogen) using random hexamer primers. Expression of the influenza matrix (M1) gene was determined by quantitative real time PCR (qRT-PCR) as described [213]. Fluorescent reporters were detected using Bio-Rad (Hercules, CA) iCycler PCR machine and primers and probes were purchased from Applied Biosystems. The levels of mRNA for GAPDH were determined for all samples and were used to normalize expression of the influenza M1 gene. Data were converted to HAU units using real time PCR standards made from the virus stock with known HAU titer.

Quantitation of lung mRNA cytokine levels. mRNA levels for murine RANTES, MCP-1, IL-12, IL-1β, TNF-α and GAPDH were determined using qRT-PCR, as described above. All data are expressed as the ratio to vitamin C supplemented, uninfected controls.

Statistical analysis. Ascorbic acid and glutathione data were analyzed by two-way ANOVA followed by Tukey HSD test. Real time PCR data between vitamin C deficient and vitamin C adequate groups were analyzed by Kruskal-Wallis test. Lung pathology data and Western blot quantification were analyzed by student t-test. All statistical analyses were performed with JMP software (SAS, Cary, NC). $P < 0.05$ was considered significant.

D. RESULTS

Decreased ascorbic acid concentration in unsupplemented *gulo-/-* mice. There were no differences in body weight between supplemented and unsupplemented *gulo-/-* mice, either before or after influenza infection (data not shown). Lung and liver ascorbic acid concentration were significantly lower in unsupplemented *gulo -/-* mice (2.5 percent of the control in the lung and 12 percent of the control in the liver) compared with 330 mg/L ascorbic acid supplemented *gulo -/-* mice (**figure 3.1**). Influenza infection of supplemented mice did not result in alterations in vitamin C levels. In vitamin C adequate mice, the lung had a higher concentration of vitamin C than the liver, most likely reflecting the high oxidative load present in this organ. However, in vitamin C deficient mice, vitamin C concentration in the lung dropped to a level lower than the vitamin C concentration in the liver, suggesting that the lung is more susceptible to vitamin C deficiency when compared with the liver. Vitamin C levels did not show sex differences between male and female mice (data not shown).

Increased total and reduced glutathione (GSH) in vitamin C deficient mice. At baseline and day 1 post infection, vitamin C deficient mice had an increase in the reduced form of glutathione (GSH) and in total glutathione in the lung compared with vitamin C adequate mice (**table 3.1**). The oxidized form of glutathione (GSSG) and the reduced/oxidized glutathione ratio (GSH/GSSG) did not differ between vitamin C adequate and deficient mice (data not shown). Glutathione levels did not differ between male and female mice (data not shown). It is likely that vitamin C deficient mice were synthesizing more glutathione in the lung to compensate for the vitamin C deficiency.

Increased lung pathology of vitamin C deficient mice. At day 7 post infection, vitamin C deficient mice had significantly greater lung pathology compared with vitamin C adequate mice. Interestingly, the vitamin C deficient mice had less lung pathology early post-infection, at day 1 and 3. There was no difference in pathology between male and female mice (data not shown) (**figure 3.2**).

Lung influenza virus titer. Influenza virus titers in the lung peaked at day 1 post infection (about 300 HAU), and declined steadily in both vitamin C adequate and deficient mice. There were no differences between groups or between male and female (data not shown).

Lung proinflammatory chemokine expression. There was a sex difference on the effect of vitamin C deficiency on the expression of mRNA for chemokines in the lungs of infected mice. Male vitamin C deficient *gulo* -/- mice had a lower expression of RANTES mRNA in the lung at 1 day post infection and a higher expression of RANTES and MCP-1 mRNA at day 3 post infection compared with male vitamin C adequate *gulo* -/- mice. None of these differences were observed in female vitamin C deficient mice (**figure 3.3**).

Lung proinflammatory cytokine expression. There was a sex difference in the effect of vitamin C deficiency on the expression of mRNA for pro-inflammatory cytokines in the lungs of infected mice. Male vitamin C deficient *gulo* -/- mice had a lower expression of mRNA for IL-1β and TNF-α in the lungs 1 day post-infection. There was a higher expression of mRNA for IL-12 by vitamin C deficient mice at day 3 post-infection. Overall, vitamin C adequate male mice had a rapid immune response which appeared to be delayed in vitamin C deficient male mice. None of these differences were observed in female vitamin C deficient mice (**figure 3.4**).

Lung NF-κB activation. Because NF-κB plays a critical role in the inflammatory response and vitamin C has been shown to regulate NF-κB activation [170], we further investigated whether the difference in chemokine and cytokine expression in male *gulo-/-* mice was mediated by a difference in NF-κB activation. At day 1 post-infection, male vitamin C deficient mice had a lower level of IκBα in the cytosolic fractions and a higher level of NF-κB in the nuclear fractions of cells in the lung, indicating that vitamin C deficiency resulted in NF-κB over-activation in the lung in response to influenza virus infection (**figure 3.5**).

E. DISCUSSION

Influenza virus infection in mice is characterized by a rapid inflammatory response in the lung. It has been reported that nitric oxide (NO) and other reactive oxygen intermediates (ROI) are involved in the pathogenesis of influenza virus-induced pneumonia [233]. In addition to their ability to cause direct tissue damage, there is considerable evidence implicating the role of ROI in cellular signaling and transcriptional regulation [68, 69]. ROI regulate the immune response through redox-sensitive transcription factors like NF-κB [70]. Hydrogen peroxide activates NF-κB by increasing the phosphorylation of MAPK p38 and Akt, components of the protein kinase cascades that lead to IκB phosphorylation [69].

This study describes for the first time the immune response to infection with influenza virus in *gulo-/-* vitamin C deficient mice. We found that vitamin C deficient mice had greater lung pathology late post-infection compared with vitamin C adequate controls, although there were no differences in lung viral titers. These results suggest that vitamin C does play a role in reducing influenza virus induced lung pathology.

A main contributor to influenza-induced lung pathology is the infiltration of inflammatory cells into the lungs. These immune cells produce a variety of chemokines and cytokines that are involved in attracting increased numbers and activation of immune cells to combat the viral infection. Chemokines are potent chemoattractant cytokines and have been considered the main candidate molecules responsible for the selective recruitment of distinct leukocyte populations. Member of the CC-chemokine subfamily, such as MCP-1 and RANTES, preferentially attract monocytes and lymphocytes [25]. RANTES is produced by CD8+ T cells, epithelial cells, fibroblasts and platelets and plays a key role in the immune response to viral infection [26]. MCP-1 is produced by monocytes, macrophages and

fibroblasts. MCP-1 has inflammatory properties similar to RANTES in terms of recruiting monocytes and lymphocytes. RANTES and MCP-1 are both involved in several inflammatory disorders of the lung [28, 29].

To determine if vitamin C deficiency could alter the production of cytokines/chemokines by immune cells, we examined the lungs of infected mice for pro-inflammatory cytokine and chemokine mRNA levels. At day 1 post infection, vitamin C deficient male mice had a higher expression of mRNA for RANTES compared with vitamin C adequate male mice. However, at day 3 post infection, vitamin C deficient male mice had less RANTES and MCP-1 mRNA production in the lungs. Female vitamin C deficient mice had RANTES and MCP-1 mRNA levels similar to vitamin C adequate female mice. Thus, male mice deficient in vitamin C had an impaired immune response to influenza infection, which may have contributed to increased lung pathology.

Examination of pro-inflammatory cytokines IL-12, IL-1β, and TNF-α also revealed sex differences. IL-12 is considered a central regulator of immune responses [234]. Produced by dendritic cells, macrophages and neutrophils, IL-12 promotes natural killer (NK) cells and cytotoxic T lymphocyte activity as well as inducing the secretion of IFN-γ by NK cells [234]. IL-12 mRNA levels were higher at day 3 in male vitamin C deficient mice compared with controls. Female vitamin C deficient mice had a trend towards a higher IL-12 mRNA production at day 7 as well ($P = 0.077$). The higher IL-12 levels in vitamin C deficient mice may be a consequence of increased inflammatory cells present in the lungs of these mice.

IL-1β and TNF-α are also pro-inflammatory cytokines. Produced by activated macrophages and monocytes, IL-1β generates systemic and local responses to infection by inducing fever, activating lymphocytes and promoting migration of leukocytes into the site of

infection [235]. TNF-α is also produced largely by macrophages and monocytes. TNF-α induces a local protective effect by acting on blood vessels to increase vascular permeability to fluid, proteins, and cells, and increasing endothelial adhesiveness for leukocytes and platelets [39]. Only male *gulo-/-* mice demonstrated a difference in mRNA for IL-1β and TNF-α between vitamin C deficient and vitamin C supplemented groups. At day 1 post infection, mRNA for both of the cytokines were lower in the vitamin C deficient male mice compared with vitamin C adequate male mice, suggesting that the less lung pathology at day 1 post infection may be due to lower levels of inflammatory mediators. However, because female *gulo-/-* mice also had less pathology at day 1 post infection without the corresponding lower levels of IL-1β and TNF-α, there are likely other inflammatory mediators that contributed to the inflammatory process.

The delay in the development of pathology in the vitamin C deficient mice may have been due to a slow initial immune response in these animals. The less immune cell infiltration of the vitamin C deficient mice at 1 and 3 days post infection may be due to less production of chemokines and cytokines at early stage in the infection as well as changes in the migration ability of inflammatory cells. For example, Ganguly et al. [165] demonstrated a significant reduction in the migration of macrophages *in vitro* from vitamin C deficient guinea pigs compared with vitamin C sufficient guinea pigs. Addition of vitamin C to the cultures partially reversed the reduced migration [165].

How does a deficiency in vitamin C affect the expression of mRNA for cytokines and chemokines? One possibility is the greater oxidative stress in the vitamin C deficient mice. Alterations in GSH and GSSG levels in the vitamin C deficient mice suggest that these mice were oxidatively stressed. Higher levels of ROI are associated with NF-κB activation [130,

236]. NF-κB, in turn, activates gene transcription for a number of cytokines and chemokines including IL-12, RANTES and MCP-1. We found that lungs from vitamin C deficient male mice had a lower level of the NF-κB inhibitor, IκB in the cytosol, and a higher level of NF-κB in the nucleus compared with vitamin C adequate male mice. These results suggest that the greater oxidative stress in the vitamin C deficient male mice following influenza infection led to greater NF-κB activation, which in turn upregulated the transcription of pro-inflammatory cytokines and chemokines. Female vitamin C deficient mice also tended to have a higher level of NF-κB activation (Data not shown), likely contributing to the greater lung pathology in female vitamin C deficient mice. However, because no differences in pro-inflammatory mediators were detected in female vitamin C deficient mice, other possibilities for the greater pathology must be considered, for example, there may be a difference in chemokine receptor expression between male and female.

Vitamin C deficiency did not affect the ability of the immune system to eliminate influenza virus replication in the lungs, reflected by similar viral titers between vitamin C deficient and supplemented mice. However, influenza A/Bangkok/1/79 is a mild strain of a human influenza virus which does not result in lethal infection when inoculated into mice. Because this strain of influenza virus is not mouse-adapted, it induces a mild lung pathology from which the mice recover. Although a deficiency in vitamin C did not inhibit influenza virus clearance in this study, the possibility that vitamin C deficiency could impair the ability of the host to clear a more virulent mouse-adapted strain of influenza virus can not be excluded.

This study also demonstrated that vitamin C deficiency affected the immune response of male, but not female, mice. Lung and liver vitamin C levels were not different between

male and female *gulo -/-* mice, suggesting that the sex difference was not due to different tissue vitamin C concentrations. However, we can not rule out the possibility that immune cell vitamin C concentrations differed between males and females. Sex differences in immune responses are known. Males and females have differences in immune responses to injury and infection, and the gonadal steroid hormones, estrogen and testosterone, are responsible for the differences [237-239]. Physiological levels of estrogen stimulates the immure response while testosterone suppresses it [240-242]. A more recent *ex vivo* study using whole blood of humans found that men had a higher percentage of TNF-α, IL-1β, and IL-12 producing monocytes compared with women after endotoxin stimulation [243]. However, because the pathology in our study was similar between male and female vitamin C deficient mice despite the clear immunological differences, it is likely that other immune functions may have been impaired in the female mice, such as NK cell activity or different pro-inflammatory mediators that were not examined in this study. To our knowledge, this study is the first to demonstrate a sex difference in the effect of vitamin C deficiency on influenza infection.

We conclude that vitamin C is an important nutrient for adequate immune function and limiting pathogenesis following influenza virus infection. As shown for other antioxidant nutrients, such as selenium and vitamin E, vitamin C may be important in limiting the increased oxidative stress that occurs during an influenza infection, thus lowering tissue inflammation. Further studies characterizing the sex effect of vitamin C are warranted.

Table 3.1: Lung total GS, GSH, GSSG levels and GSH/GSSG ratios in vitamin C adequate and vitamin C deficient mice[1]

	Vit.C status	Total GS	GSH	GSSG	GSH/GSSG
d 0	Adequate	4.28 ± 0.09	4.03 ± 0.09	0.13 ± 0.01	32.12 ± 2.04
	Deficient	4.79 ± 0.05	4.50 ± 0.07	0.15 ± 0.01	31.58 ± 1.97
	P-value[2]	< 0.05	< 0.05	NS	NS
d 1	Adequate	4.55 ± 0.12	4.29 ± 0.13	0.13 ± 0.01	35.88 ± 3.49
	Deficient	5.12 ± 0.15	4.83 ± 0.14	0.14 ± 0.01	36.88 ± 4.19
	P-value	< 0.05	< 0.05	NS	NS
d 3	Adequate	4.41 ± 0.09	4.14 ± 0.10	0.14 ± 0.02	30.97 ± 4.61
	Deficient	4.64 ± 0.16	4.36 ± 0.16	0.14 ± 0.02	34.55 ± 3.74
	P-value	NS	NS	NS	NS

[1] Values are expressed as mean ± S.E.
[2] P-Values are from two-way ANOVA; the effect of day was NS, $P > 0.05$.

Figure 3.1: Ascorbic acid (reduced form of vitamin C) levels in lung and liver from gulo -/- mice prior to influenza virus infection (day 0), and at 1, 3, and 7 days post infection. *Gulo* -/- mice were provided with water or water containing 330 mg/L vitamin C. Data are expressed as mean ± S.E. n = 4-5. Asterisks indicate significant difference between unsupplemented group and 330 mg/L ascorbic acid supplemented group (P < 0.05).

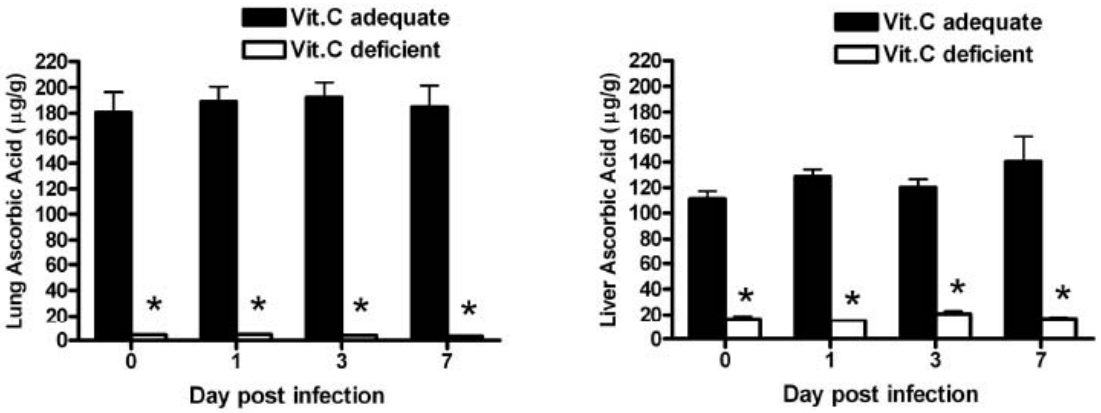


Figure 3.2: Lung pathology of vitamin C adequate and vitamin C deficient gulo-/- mice prior to influenza virus infection (day 0), and at 1, 3, and 7 days post infection. Data are expressed as mean ± S.E. n = 5-10. Asterisks indicate significant difference between vitamin C adequate and deficient groups (P < 0.05).

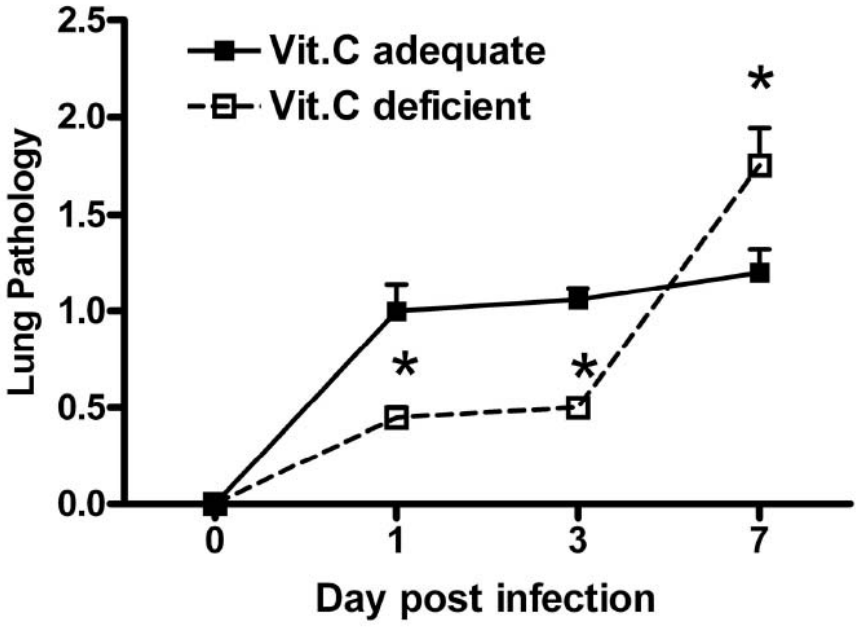


Figure 3.3: Lung mRNA levels for RANTES and MCP-1 from vitamin C adequate and deficient gulo-/- mice prior to influenza virus infection (day 0), and at 1, 3, and 7 days post infection. Data are expressed as the ratio to the day 0 levels of vitamin C adequate group ± S.E. n = 4-5. Asterisks indicate significant difference between vitamin C adequate and vitamin C deficient groups (P < 0.05).

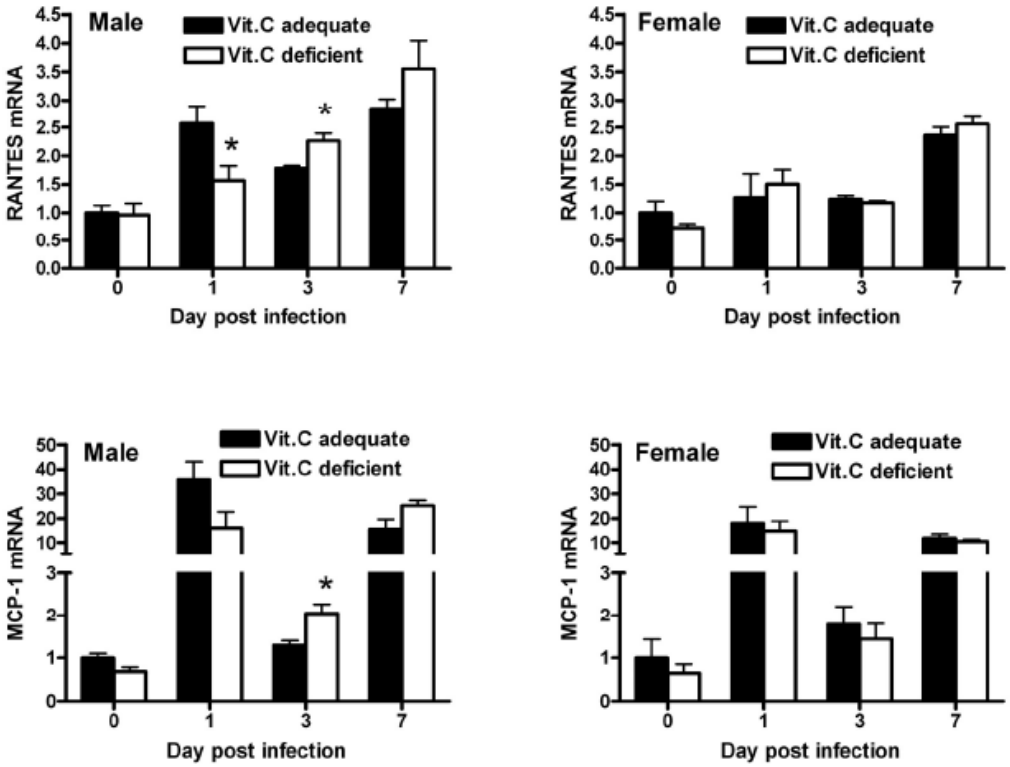


Figure 3.4: Lung mRNA levels for IL-12, IL-1β, and TNF-α from vitamin C adequate and deficient gulo-/- mice prior to influenza virus infection (day 0), and at 1, 3, and 7 days post infection. Data are expressed as the ratio to the day 0 levels of vitamin C adequate group ± S.E. n = 4-5. Asterisks indicate significant difference between vitamin C adequate and vitamin C deficient groups (P < 0.05).

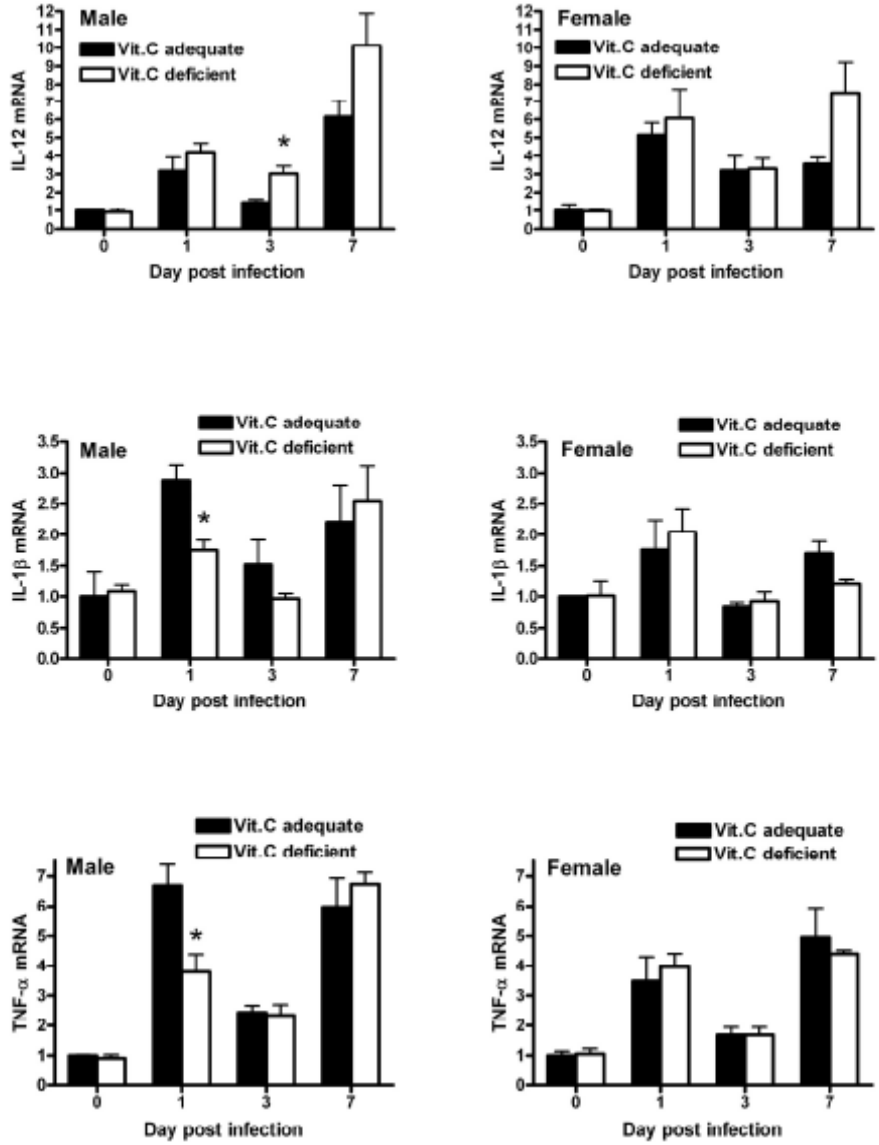
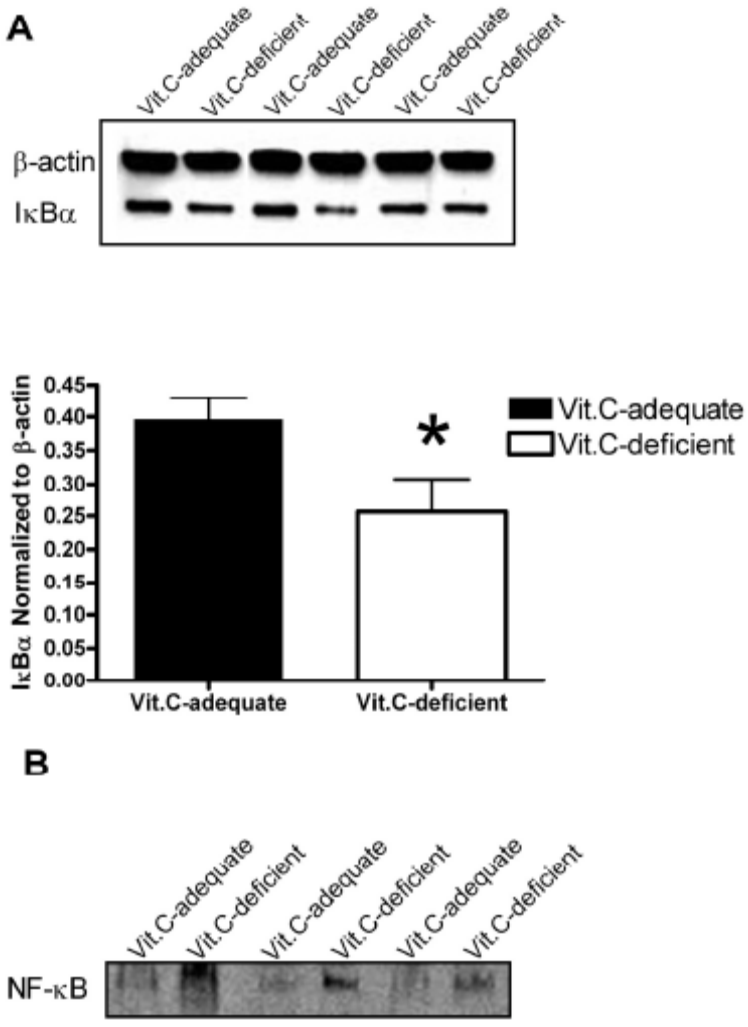


Figure 3.5: Male vitamin C deficient mice have increased NF-κB activation in the lung at day 1 post influenza virus infection. (A) IκBα in the cytosolic fractions of the lung was measured by Western blotting. Densitometric analysis was conducted on immunoblots, and results are expressed as the ratio to β-actin ± S.E. The asterisk indicates significant difference (P < 0.05) (B) NF-κB DNA binding was measured in the nuclear fractions of the lung by EMSA.



Chapter IV

Influence of vitamin C on the response to influenza A/PR8/34 infection

A. ABSTRACT

This study was designed to determine the effect of vitamin C deficiency on the immune response to infection with a virulent strain of influenza virus. Vitamin C deficient male *gulo-/-* mice had a higher lung viral titer at day 7 post-infection compared with vitamin C adequate mice. Correlating with the viral titer, lung mRNA expression for IL-1β was also higher in vitamin C deficient male mice. In contrast, no difference in viral titer or IL-1β was observed in females. There was no difference in RANTES, IL-12 and TNF-α expression between vitamin C deficient and vitamin C adequate groups in either male or female mice. These data suggest that an adequate vitamin C intake is essential for the eradication of a virulent strain of influenza virus and that there is indeed a sex difference of the effect of vitamin C deficiency on the immune response to influenza infection.

B. INTRODUCTION

Previous studies have demonstrated that vitamin C deficient *gulo-/-* mice had an altered immune response to influenza A/Bangkok/1/79 infection. At day 7 post-infection, vitamin C deficient mice had significantly increased lung pathology compared with vitamin C adequate mice. However, vitamin C deficient mice had less lung pathology early post-infection, at day 1 and 3. Although there was no difference in lung pathology between male and female mice, only vitamin C deficient male *gulo-/-* mice had altered chemokine and cytokine profiles post infection compared with male vitamin C adequate controls. Measurement of lung influenza viral titer showed no difference between vitamin C adequate and vitamin C deficient groups, either male or female.

Influenza A/Bangkok/1/79 is a mild strain of human influenza virus which does not result in a lethal infection when inoculated into mice. Because this strain of influenza virus is not mouse-adapted, it induces a mild lung pathology from which mice readily recover. Although a deficiency in vitamin C did not affect virus eradication in the previous study, we could not exclude the possibility that vitamin C deficiency may impair the ability of the host to eradicate a virulent strain of influenza virus. To test this hypothesis, we infected vitamin C deficient *gulo-/-* mice with influenza A/PR8/34 (PR8), a mouse-adapted strain of influenza which replicates efficiently in mice [208], causes a severe inflammatory response [45], and can result in mortality. We measured lung influenza viral titers and the expression of chemokines/cytokines post-infection.

C. MATERIALS AND METHODS

Influenza virus. Influenza A/Puerto Rico/8/34 was propagated in 10-day-old embryonated hen's eggs [210]. The virus-containing allantoic fluid was collected and stored at -80°C. This mouse-adapted strain of influenza virus causes a strong inflammatory response in normal mice [45].

Mice. *Gulo-/-* mice are as previously described [171]. Five-week old male and female *gulo-/-* mice were provided with water or water containing 330 mg/L vitamin C for three weeks prior to inoculation with influenza PR8. At day 7 post-infection, mice were killed and the lungs were collected for the determination of influenza viral titer and the expression of proinflammatory cytokines and chemokines.

Quantitation of viral titer by real time PCR. To determine lung viral titers, half of the right lung was removed, and total RNA was isolated using the TRIzol method (Invitrogen). Reverse transcription was carried out using Superscript II First Strand Synthesis kit (11904-018, Invitrogen) using random hexamer primers. Expression of the influenza matrix (M1) gene and GAPDH were determined by quantitative real time PCR (qRT-PCR) as described [213]. Fluorescent reporters were detected using Bio-Rad (Hercules, CA) iCycler PCR machine and primers and probes were obtained from Applied Biosystems (Foster City, CA). The levels of mRNA for GAPDH were determined for all samples and were used to normalize expression of the influenza M1 gene. Data were converted to hemagglutination units (HAU) using real time PCR standards made from the virus stock with known HAU titer.

Quantitation of lung chemokine and cytokine mRNA levels. mRNA levels for murine RANTES, IL-1β, IL-12 and TNF-α were determined using qRT-PCR, as described

above. All data are expressed as the ratio to the mean of vitamin C adequate male and female groups combined.

Statistical analysis. Data were analyzed by Kruskal-Wallis test with JMP software (SAS, Cary, NC).

D. RESULTS

Lung influenza viral titer. At day 7 post infection, vitamin C deficient male mice had a significantly higher influenza viral titer in the lung compared with vitamin C adequate male mice. There was no difference in lung viral titers between vitamin C adequate and vitamin C deficient female mice. When viral titers were compared between male and female within vitamin C adequate or vitamin C deficient groups, vitamin C deficient male mice had a significantly higher viral titer compared with female vitamin C deficient mice (figure 4.1). There was no difference in lung viral titers between male and female vitamin C adequate mice.

Lung chemokine/cytokine expression. At day 7 post infection, vitamin C deficient male mice had significantly higher mRNA levels for IL-1β in the lung compared with vitamin C adequate male mice. No difference was observed in IL-1β expression in female mice (figure 4.2). The mRNA levels for RANTES, IL-12 and TNF-α were also measured with no differences between vitamin C adequate and vitamin C deficient groups or between male and female (data not shown).

E. DISCUSSION

This study demonstrated that the eradication of a virulent strain of influenza virus was impaired in vitamin C deficient *gulo-/-* mice. In our previous study, the immune response to a mild strain of influenza virus was altered in vitamin C deficient *gulo-/-* mice compared with vitamin C adequate *gulo-/-* mice. The effect of vitamin C deficiency on the immune response also had a sex difference: male *gulo-/-* mice appeared to be more susceptible to vitamin C deficiency compared with females. Although vitamin C deficiency did not affect lung viral titer after infection with a mild strain of influenza virus, we hypothesized that the altered immune response in vitamin C deficient mice may result in an impaired eradication of a virulent strain of influenza virus.

Unlike influenza A/1/79, which is a mild strain of human influenza and is not adapted to grow efficiently in mice, influenza A/PR8/34 (PR8) is a mouse-adapted strain of influenza. Influenza PR8 grows efficiently in normal mice [208] and causes a strong inflammatory response [45]. At day 7 post influenza PR8 infection, vitamin C deficient male mice had 2 times the lung viral titer compared with vitamin C adequate male mice. There are two possible explanations for the higher viral titer in vitamin C deficient male mice. It may be that influenza virus replicates more efficiently in the vitamin C deficient male mice, or the vitamin C deficient male mice have immune dysfunction, which impairs their ability to clear the virus.

Our previous study has shown that vitamin C deficient male mice had higher NF-κB activation compared with vitamin C adequate mice. Influenza virus replication has been shown to be dependent on NF-κB activation and cells with low NF-κB activity were resistant to influenza virus infection, but became susceptible upon activation of NF-κB [244],

suggesting influenza virus may replicate more efficiently in vitamin C deficient male mice due to the higher level of NF-κB activation in these mice. On the other hand, our previous study also demonstrated that the immune response to influenza infection was delayed in vitamin C deficient mice. During the time of peak influenza A/Bangkok/1/79 titer in the lung, vitamin C deficient male mice failed to mount a full immune response compared with vitamin C adequate male mice. In the case of influenza PR8 infection, this may result in an impaired viral clearance.

RANTES is produced by CD8+ T cells, epithelial cells, fibroblasts and platelets and plays a key role in the immune response to viral infection [26]. IL-12 is considered a central regulator of immune responses [234]. Produced by dendritic cells, macrophages and neutrophils, IL-12 promotes natural killer (NK) cells and cytotoxic T lymphocyte activity as well as inducing the secretion of IFN-γ by NK cells [234]. IL-1β and TNF-α are also pro-inflammatory cytokines. Produced by activated macrophages and monocytes, IL-1β generates systemic and local responses to infection by inducing fever, activating lymphocytes and promoting migration of leukocytes into the site of infection [235]. TNF-α is also produced largely by macrophages and monocytes. TNF-α induces a local protective effect by acting on blood vessels to increase vascular permeability to fluid, proteins, and cells, and increasing endothelial adhesiveness for leukocytes and platelets [39]. At day 7 post-infection, although the lung viral titer in vitamin C deficient male mice was one time higher than vitamin C adequate male mice, RANTES, IL-12 and TNF-α expression had no difference except for the expression of IL-1β, again suggesting the lack of responsiveness to influenza infection in vitamin C deficient male mice.

After influenza PR8 infection, the difference in lung viral titer was only seen in male mice. Female mice had similar lung viral titers between vitamin C adequate and vitamin C deficient groups. The male vitamin C deficient mice also had a significantly higher lung viral titer compared with female vitamin C deficient mice. The sex difference in the current study complements our previous observations that male *gulo-/-* mice were more susceptible to the effect of vitamin C deficiency than female *gulo-/-* mice.

In summary, the current study validated our hypothesis that vitamin C deficient *gulo-/-* mice would have an impaired viral clearance when infected with a virulent strain of influenza virus. The difference in lung viral titers between vitamin C adequate and vitamin C deficient groups post-infection was only seen in male mice, suggesting the effect of vitamin C deficiency on immune response to influenza infection indeed has a sex difference.

Figure 4.1: Influenza viral titers in the lung at day 7 post-infection. *Gulo*-/- mice were provided with regular water or water containing 330 mg/L. Data are expressed as mean ± S.E. Asterisks indicate significant difference (P < 0.05).

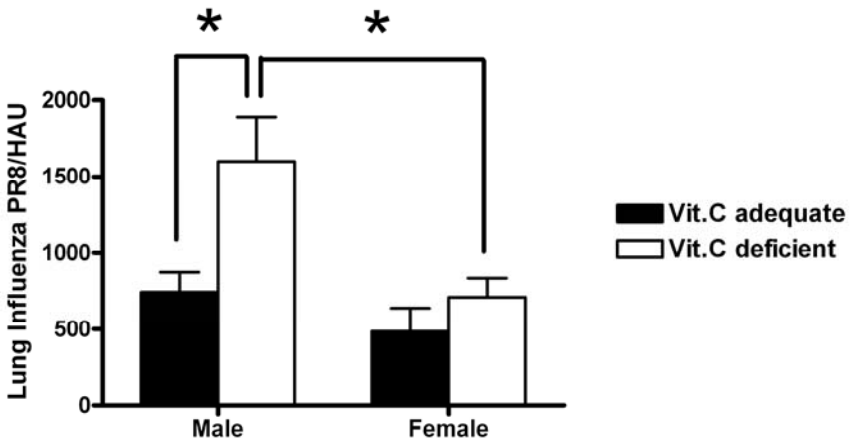
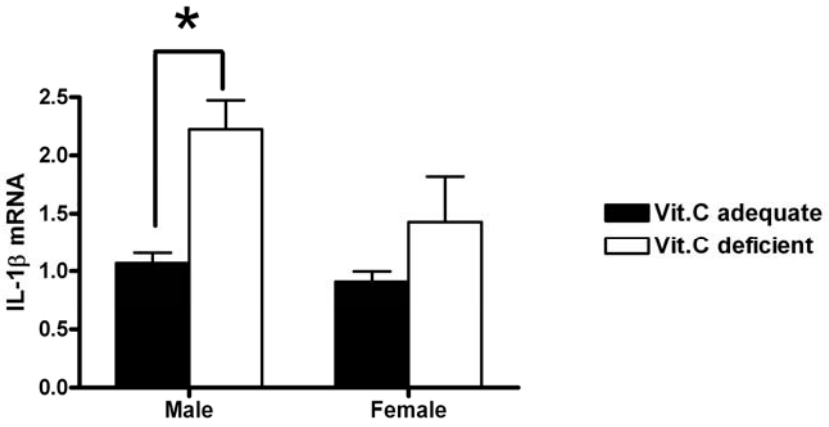


Figure 4.2: Lung mRNA levels for IL-1β from gulo-/- mice at day 7 post-infection. *Gulo*-/- mice were provided with regular water or water containing 330 mg/L vitamin C. Data are expressed as mean ± S.E. The asterisk indicates significant difference (P < 0.05).



Chapter V

The effect of a high-dose vitamin C supplementation on the immune response to influenza virus infection

A. ABSTRACT

This study was designed to examine the effect of a high-dose vitamin C supplementation on the immune response to influenza virus infection. *Gulo-/-* mice require vitamin C supplementation in their drinking water for survival. Five week old male and female *gulo-/-* mice were provided with regular water (vitamin C deficient), water containing 330 mg/L vitamin C (low-dose vitamin C) or water containing 3.3 g/L vitamin C (high-dose vitamin C) for three weeks prior to inoculation with influenza A/Bangkok/1/79. The high-dose vitamin C supplemented mice and the low-dose vitamin C supplemented mice had greater lung pathology at 1 and 3 days post-infection but had less lung pathology at day 7 post infection compared with the vitamin C deficient mice. There was no difference in lung viral titers between the high-dose vitamin C supplemented mice and the low-dose vitamin C supplemented mice or between the high-dose vitamin C supplemented mice and the vitamin C deficient mice. The high-dose vitamin C supplemented mice had significantly higher levels of IFN-γ expression post-infection compared with the vitamin C deficient mice and higher levels of RANTES expression post-infection compared with the low-dose vitamin C supplemented mice. The high-dose vitamin C supplemented female mice also had higher levels of MIP-1α and TNF-α expression at day 7 post-infection compared with the vitamin C deficient mice. The high-dose vitamin C supplementation did not down-regulate the viral infection-induced NF-κB activation in male *gulo-/-* mice. Taken together, these data suggest that a high-dose vitamin C supplementation is effective in limiting lung pathology and enhancing the Th1 type post influenza infection, but a high-dose vitamin C supplementation is not as effective as a low-dose supplementation in down-regulating the influenza-stimulated NF-κB activation.

B. INTRODUCTION

Previous studies in our laboratory have demonstrated that the immune response to influenza A/Bangkok/1/79 infection was altered in the vitamin C deficient *gulo-/-* mice compared with 330 mg/L vitamin C supplemented *gulo-/-* mice. The vitamin C deficient mice had greater lung pathology and an over-activation of NF-κB post-infection. The effect of vitamin C deficiency on the immune response to influenza infection also had a clear sex difference: in the male mice, vitamin C deficiency resulted in an altered chemokine and cytokine expression profile while no effect of vitamin C deficiency on chemokine/cytokine expression was observed in the female mice. These data suggest that the 330 mg/L vitamin C supplementation in drinking water is beneficial during influenza virus infection by optimizing the immune response and protecting the lung from oxidative stress induced damage.

However, 330 mg/L vitamin C in the drinking water is a relatively low-dose supplementation. The plasma vitamin C concentration in 330 mg/L vitamin C supplemented mice is 60% of wild-type mice [171]. Therefore the previous study raised a question: will a high-dose vitamin C supplementation further alter the immune response to influenza infection and have more beneficial effects?

In human studies, high-dose vitamin C supplementations have been used in the treatment of inflammatory diseases and in some cancer prevention studies. Although the effect of high-dose vitamin C supplementation on common colds has been controversial for decades, little is known about the effect of the high-dose vitamin C supplementation on influenza virus infection. In the current study, we supplemented the *gulo-/-* mice with high-dose vitamin C (3.3 g/L) in the drinking water prior to influenza infection. The effects of the

high-dose vitamin C supplementation on influenza infection were compared with those of the low-dose vitamin C supplementation and vitamin C deficiency.

C. METERIALS AND METHODS

Influenza virus. Influenza A/Bangkok/1/79 was used for inoculation of mice as described in the previous study.

Mice. *Gulo-/-* mice are as previously described. Five-week old male and female *gulo-/-* mice were provided with water, water containing 330 mg/L vitamin C or water containing 3.3 g/L vitamin C for three weeks prior to inoculation with influenza A/Bangkok/1/79. At day 1, 3 and 7 post-infection, mice were killed and the lungs were collected for the determination of pathology, influenza viral titer and the expression of proinflammatory chemokines and cytokines.

Infection of mice. Mice were lightly anethetized with an intraperitoneal injection of ketamine (0.022 mg) and xylazine (0.0156 mg). Following anesthesia, mice were infected intranasally with 10 HAU (Hemagglutinating Unit) of influenza A/Bangkok/1/79 in 0.05 mL sterile PBS.

Tissue ascorbic acid measurement. Ascorbic acid (the reduced form of vitamin C) was measured by the α,α'-dipyridyl method as described [232].

Histopathology of lungs. The left lung was removed and inflated with 4% paraformaldehyde fixative in 0.1M Na phosphate buffer (pH 7.2). Sections (6 μm) were fixed in acetone and stained with hematoxylin-eosin. The extent of inflammation was graded without knowledge of the experimental variables by two independent investigators. Grading was performed semiquantitatively according to the relative degree (from lung to lung) of inflammatory infiltration. The scoring was as follows: 0, no inflammation; 1+, mild influx of inflammatory cells with inflammatory infiltrates clustered around vessels; 2+, increased

inflammation with approximately 25-50% of the total lung involved; 3+, severe inflammation involving 50-70% of the lung; and 4+, almost all lung tissue contains inflammatory infiltrates.

Western blot. Cytosol and nuclear protein extracts were prepared from lung tissue using Nuclear/Cytosol Fractionation Kit (K266-100, Biovision, Mountain View, CA) following protocols provided by the manufacturer. Extracts were separated by 12% (wt/vol) sodium dodecyl sulfate (SDS) polyacrylamide gels and electrophoretically blotted onto Immobilon-P membranes (Millipore) according to standard procedures. The membranes were blocked with 5% non-fat milk and treated with rabbit anti-mouse IκBα (9242, Cell Signaling Technology, Danvers, MA), anti-mouse NF-κBp65 (sc-109, Santa Cruz Biotechnology, Santa Cruz, CA), or anti-mouse β-actin (4967, Cell Signaling Technology, Danvers, MA) overnight at 4°C. Membranes were then incubated with a secondary goat anti-rabbit IgG conjugated with horseradish peroxidase (7074, Cell Signaling Technology, Danvers, MA) for 1h at room temperature and stained with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). The chemiluminescent signals were acquired using a 16-bit CCD camera (GeneGnome system; Syngene, Frederick, MD). Densitometry analysis was conduct using the GeneSnap software (Syngene).

Electrophoretic mobility shift assay (EMSA). EMSA was performed with the Panomics' EMSA Kit (AY1030, Panomics, Redwood City, CA) according to protocols provided by the manufacturer.

Quantitation of viral titers. Half of the lung was removed, and total RNA was isolated using the TRIzol method (Invitrogen). Reverse transcription was carried out using Superscript II First Strand Synthesis kit (11904-018, Invitrogen) using random hexamer primers. Expression of the influenza matrix (M1) gene was determined by quantitative real

time PCR (qRT-PCR) as described [213]. Fluorescent reporters were detected using Bio-Rad (Hercules, CA) iCycler PCR machine and primers and probes were purchased from Applied Biosystems. The levels of mRNA for GAPDH were determined for all samples and were used to normalize expression of the influenza M1 gene. Data were converted to HAU units using real time PCR standards made from the virus stock with known HAU titer.

Quantitation of lung mRNA cytokine levels. mRNA levels for murine IFN-α, IFN-β, IFN-γ, RANTES, MCP-1α, MIP-1, IL-12, IL-1β, TNF-α, IL-6, IL-10 and GAPDH were determined using qRT-PCR, as described above. All data are expressed as the ratio to vitamin C supplemented, uninfected controls.

Immunohistochemistry. Lungs were inflation fixed with 4% paraformaldehyde overnight at 4°C and transferred to PBS and processed for paraffin embedding. We cut 4-pm sections using a Leica RM 2155 microtome (Leica, Deerfield, IL) onto Fisher Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Sections were deparaffinized by heating at 60°C overnight and washing twice with xylene, rehydrated in a graded series of ethanol, and washed in Tris-buffered saline, pH 8.0 (TBS). For immunostaining, sections were washed three times for 5 min in TBS-0.1% Triton X-100 (TBST) followed by 90-min block with goat serum (Sigma). Sections were then incubated overnight at 4°C with anti-mouse NF-κBp65 (sc-109, Santa Cruz Biotechnology, Santa Cruz, CA) diluted with TBST-3% BSA. After incubation, sections were washed five times for 5 min with TBST and incubated for 1 hr with Alexa Fluor® 488 goat anti-rabbit IgG secondary antibody (Invitrogen). Sections were then washed five times for 5 min with TBST and mounted with Crystal/ Mount (Biomeda, Foster City, CA). Photographs were taken with a Nikon Coolpix 990 digital camera attached to a Nikon E600 light microscope (Nikon, Tokyo, Japan).

Statistical analysis. Ascorbic acid data were analyzed by two-way ANOVA followed by Tukey HSD test. Real time PCR data between vitamin C deficient and vitamin C adequate groups were analyzed by Kruskal-Wallis test. Lung pathology data and Western blot quantification were analyzed by student t-test. All statistical analyses were performed with JMP software (SAS, Cary, NC). $P < 0.05$ was considered significant.

D. RESULTS

Mice with high-dose vitamin C supplementation had higher levels of vitamin C in the liver. At baseline prior to influenza virus infection (day 0) and at 1 and 3 days post-infection, *gulo-/-* mice supplemented with 3.3 g/L vitamin C had significantly higher levels of ascorbic acid in the liver compared with *gulo-/-* mice supplemented with 330 mg/L vitamin C. There was no difference in ascorbic acid levels in the lung between the high-dose and the low-dose supplemented groups. As expected, the high-dose and the low-dose vitamin C supplemented groups had significantly higher levels of ascorbic acid in the lung and the liver compared with the vitamin C deficient group (figure 5.1).

The high-dose and the low-dose vitamin C supplementations reduced influenza-induced lung pathology. At 1 and 3 days post-infection, the high-dose and the low-dose vitamin C supplemented mice had significantly greater lung pathology compared with the vitamin C deficient mice. However, at day 7 post-infection, both vitamin C supplemented groups had significantly less lung pathology compared with the vitamin C deficient group. There was no difference in the lung pathology between the high-dose vitamin C supplemented group and the low-dose vitamin C supplemented group, suggesting a higher dose of vitamin C supplementation did not further reduce the lung pathology (figure 5.2).

Lung influenza viral titers unaffected by vitamin C status. There was no stitastical difference in lung viral titers among the 3 groups. The viral titers peaked 1 day post-infection and started to decline at day 3. At day 7 post-infection, the virus was barely detectable in the lungs from all groups (figure 5.3).

IFN-α and IFN-β mRNA expression not affected by vitamin C status. Lung mRNA levels for the antiviral interferons IFN-α and IFN-β were measured in male *gulo-/-*

mice. Lung mRNA levels for IFN-α (figure 5.4 A) and IFN-β (figure 5.4 B) were not different among groups. Also consistent with the viral titers, the IFN-α and IFN-β expression peaked 1 day post-infection and steadily declined after day 1.

High dose vitamin C supplementation induces increased IFN-γ expression post influenza infection. At day 3 post-infection in both male and female mice and at day 7 post infection in female mice, the high-dose vitamin C supplemented group had significantly higher lung mRNA levels for IFN-γ compared with the vitamin C deficient group. No difference was observed between the low-dose supplemented group and the deficient group, or between the high-dose supplemented group and the low-dose supplemented group (figure 5.5).

Effect of vitamin C on proinflammatory chemokine expression.. Previously, we found that low dose vitamin C supplemented male *gulo* -/- mice had a higher expression of RANTES mRNA in the lung at 1 day post infection and a lower expression of RANTES and MCP-1 mRNA at day 3 post infection compared with vitamin C deficient male *gulo* -/- mice. For the high-dose vitamin C supplemented mice, RANTES expression was significantly higher at day 3 post-infection in the male mice (figure 5.6 A) and at day 7 post-infection in the female mice (figure 5.6 B) compared with the low-dose vitamin C supplemented group. Interestingly, there was no difference in RANTES expression between the high-dose vitamin C supplemented group and the vitamin C deficient group in either male or female mice (figure 5.6 A and B).

High-dose vitamin C supplementation had no effect on lung expression of mRNA for MCP-1 at compared with either low-dose vitamin C supplemented mice or vitamin C deficient mice (figure 5.6 C and D).

In the female mice prior to infection, the high-dose vitamin C supplemented group had a significantly lower MIP-1α expression compared with either the low-dose vitamin C supplemented group or the vitamin C deficient group. However, at day 7 post-infection, the high-dose vitamin C supplemented female mice had a significantly higher MIP-1α expression compared with the vitamin C deficient group (figure 5.6 F). These difference were not found in male mice.

Effect of vitamin C on proinflammatory cytokine expression. Previously we found that low-dose vitamin C supplemented male *gulo* -/- mice had a higher expression of mRNA for IL-1β and TNF-α in the lungs 1 day post-infection and a lower expression of mRNA for IL-12 at day 3 post-infection compared with vitamin C deficient male mice. In the current study, TNF-α mRNA levels in the high-dose vitamin C supplemented female mice was significantly higher at day 7 post-infection compared with the vitamin C deficient female mice (figure 5.7 F). High-dose vitamin C supplementation did not result in differences in other cytokines measured, including IL-12, IL-1β and IL-6 compared with either the low-dose vitamin C supplemented group or the vitamin C deficient group (figure 5.7 A-E, G, H).

High-dose vitamin C supplementation had no effect on IL-10 expression post-infection. There was a significant difference in IL-10 expression between low-dose vitamin C supplemented and vitamin C deficient groups at day 1 post-infection in male mice. However, the high-dose vitamin C supplemented group had no difference in IL-10 expression compared with the vitamin C deficient group or the low-dose vitamin C supplemented group (figure 5.8).

Effect of vitamin C supplementation on nuclear NF-κB p65 protein. The amount of NF-κB p65 protein in the nuclear fractions of the lungs from vitamin C deficient, low-dose

vitamin C supplemented and high-dose vitamin C supplemented mice 1 day post influenza infection was determined by Western blot (figure 5.9 A, B and C). Vitamin C supplementation appeared to reduce the localization of NF-κB p65 into the nuclei. The high-dose supplemented mice had the lowest amount of nuclear p65 in either males or females. The difference was significant between the high-dose vitamin C supplemented group and the vitamin C deficient group in female mice (figure 5.9 D and E).

NF-κB p65 nuclear translocation was determined by immunohistochemistry. Figure 4.10 shows three representative images of p65 immunohistochemistry from the vitamin C deficient mice (figure 5.10 A), the low-dose vitamin C supplemented mice (figure 5.10 B) and the high-dose vitamin C supplemented mice (figure 5.10 C) 1 day post-infection. In all groups, most of p65 was still localized in the cytoplasm. There appeared to be no difference among groups.

Cytoplasmic IκBα protein and nuclear NF-κB DNA binding activity. The amount of IκBα in the cytoplasmic fractions of the lungs from the vitamin C deficient, low-dose vitamin C supplemented and high-dose vitamin C supplemented mice 1 day post-influenza infection was determined by Western blot (figure 5.11 A). The amount of IκBα in the cytoplasm was up-regulated by low-dose vitamin C supplementation. However, high-dose vitamin C supplementation did not appear to increase the cytoplasmic IκBα (figure 5.11 B), suggesting that low-dose vitamin C supplementation, but not high-dose supplementation, down-regulated NF-κB activation post influenza infection. This observation was supported by the EMSA assay of the nuclear fractions. The low-dose vitamin C supplementation down-regulated NF-κB DNA binding while high-dose vitamin C supplementation had no effect (figure 5.11 C).

E. DISCUSSION

The previous studies demonstrated that vitamin C deficient *gulo-/-* mice had an altered immune response to influenza virus infection. Supplementation with 330 mg/L vitamin C in the drinking water was beneficial by protecting the lung from inflammation-induced pathology and optimizing the immune response. In the current study, a high-dose of vitamin C supplementation was tested. High-dose vitamin C supplementations have been used in the prevention and treatment of colds, cancers, and other diseases. The efficacy of vitamin C for colds is an on-going debate. Although some studies, such as the recent study in Japan in which a high-dose vitamin C supplementation had a preventive effect on common colds [245], most studies supplementing up to 2 g daily vitamin C did not demonstrate a beneficial effect in normal populations [246].

The influenza virus is among the more than 200 viruses that are known to cause common cold symptoms. Infection with influenza virus causes a great deal of morbidity and mortality worldwide each year. In the U.S. alone, influenza virus infection results in over 36,000 deaths and 114,000 hospitalizations per year [13]. Although a high-dose vitamin C supplementation has been suggested to be effective in prevention and treatment of influenza infection, few studies have been done to support this hypothesis. During influenza infection, reactive oxygen species (ROS) are generated by activated immune cells, such as monocytes/macrophages and neutrophils. Although ROS can be used by phagocytic cells to aid in microbial killing [65], these oxidants can also cause direct damage to cellular structures. In addition, ROS production can be involved in subsequent morbidity and mortality due to the excessive activation of immune cells during an inflammatory response [66]. As an antioxidant vitamin, vitamin C is thought to be protective in this process. This is

supported by our previous study in which 330 mg/L vitamin C supplemented mice had improved immune responses and less lung pathology after influenza infection compared with the vitamin C deficient mice.

In the current study, 3.3 g/L vitamin C supplementation was used. This high-dose supplementation further increased the ascorbic acid concentration in the liver compared with the low-dose vitamin C supplemented mice. The high-vitamin C supplementation also had a trend toward further increasing the ascorbic acid concentration in the lung. However, this further increase in the tissue ascorbic acid level did not result in further decrease in lung pathology. The high-dose and the low-dose supplemented groups had similar lung pathology scores at various time points post-infection, demonstrating that high-dose vitamin C supplementation had a similar effect with the low-dose vitamin C supplementation in reducing influenza infection induced tissue damage. This result suggests that the maximum effect of reducing lung pathology is achieved at a lower lose of vitamin C and cannot be improved at a higher dose.

Similar with low-dose vitamin C supplementation, high-dose vitamin C supplementation did not improve viral eradication compared with the vitamin C deficient group. This is likely due to the fact that influenza A/1/79 is a mild strain of human influenza virus; even mice with an impaired immune response have the capacity of clearing it from their lungs.

The type I interferons IFN-α and IFN-β are produced in direct response to influenza infection. The induction of type I interferon by influenza virus infection is mediated by the recognition of endosomal single-stranded RNA (ssRNA) by toll-like receptor 7 (TLR7) [19].

The lack of difference in IFN-α and IFN-β expression among groups was most likely the result of the similar viral titers in all three groups.

IFN-γ is synthesized in response to the recognition of infected cells by activated T lymphocytes and NK cells [24]. The high-dose vitamin C supplemented mice had higher levels of IFN-γ expression at day 3 post-infection in male mice and at day 3 and 7 post-infection in female mice compared with vitamin C deficient mice, suggesting that the high-dose vitamin C supplementation increased T cell and/or NK cell activity. The high-dose vitamin C supplemented mice had higher levels of RANTES expression at day 3 post infection in males and at day 7 post-infection in females compared with the low-dose vitamin C supplemented group, suggesting they had a greater inflammatory response later post infection. IFN-γ plays an important role in regulating the balance between Th1 and Th2 cells. IFN-γ stimulates the synthesis of IL-12 by APCs [50-52], which drives CD4+ cells to become Th1 cells [53, 54]. RANTES and MIP-1α have also been correlated with a Th1 type response [59-62] and MIP-1α drives the development of Th1 cells *in vitro* [222]. Taken together, these data suggest that the high-dose vitamin C supplemented mice had an enhanced Th1 response compared with the vitamin C deficient mice or the low-dose vitamin C supplemented mice.

The NF-κB family of transcriptional factors regulate the expression of numerous cellular and viral genes and play an important role in immune and stress responses, inflammation, and apoptosis [71-74]. NF-κB is activated by a variety of inflammatory stimuli, including cytokines and viral infection [73, 75]. Oxidative stress also activates NF-κB [70]. Vitamin C has been shown to suppress TNF-α-induced NF-κB activation by inhibiting IκBα phosphorylation *in vitro* [170, 247, 248]. However, there are always concerns that vitamin C

may function as a prooxidant when being supplemented in high-doses, especially because of its potential to interact with transition metal ions [138]. Whether vitamin C acts as an antioxidant or prooxidant *in vivo* also depends on the metal ions released from damaged tissue. In an experiment with rats, a relatively high-dose of vitamin C (10 mg/kg) given at the time when extensive tissue damage was in progress aggravated the oxidative damage, while it protected against the damage when given before the initiation of the damage [249]. In our studies, both the low-dose and the high-dose vitamin C supplementations had a protective effect against the influenza-induced tissue damage, as reflected by the significantly less lung pathology at day 7 post-infection. The reduced NF-κB activation may partly contribute to the decreased lung pathology in the low-dose vitamin C supplemented mice compared with vitamin C deficient mice. However, the high-dose vitamin C supplemented mice had similar amount of IκBα in the cytoplasm and DNA-binding NF-κB in the nuclei, suggesting there are other mechanisms that contribute to the reduced lung pathology in these mice.

Compared with low-dose vitamin C supplementation, high-dose vitamin C supplementation resulted in further changes in the immune response to influenza infection, as reflected by a significant up-regulation of the Th1 type response at day 3 and 7 post-infection. Although high-dose vitamin C supplementation did not down-regulate NF-κB activation, it demonstrated a similar protective effect against influenza-induced lung pathology compared with the low-dose vitamin C supplementation.

Clearly, the ability of vitamin C to modulate the immune response to influenza virus infection is complex, with some cytokines and chemokines differently affected. A clear sex difference in the response also adds to the complexity. Further studies are required to fully elucidate the effects of vitamin C on viral infection.

Figure 5.1: Ascorbic acid (reduced form of vitamin C) levels in the lung and liver from gulo-/- mice prior to infection (day 0) and at 1, 3 and 7 days post-infection. *Gulo-/-* mice were provided with regular water, water containing 330 mg/L vitamin C or water containing 3.3 g/L vitamin C. Data are expressed as mean ± S.E. Asterisks indicate significant difference with the vitamin C deficient group (P < 0.05). § indicate significant difference with the low-vitamin C supplemented group (P < 0.05). Infection did not have an effect on lung or liver vitamin C level within vitamin C groups.

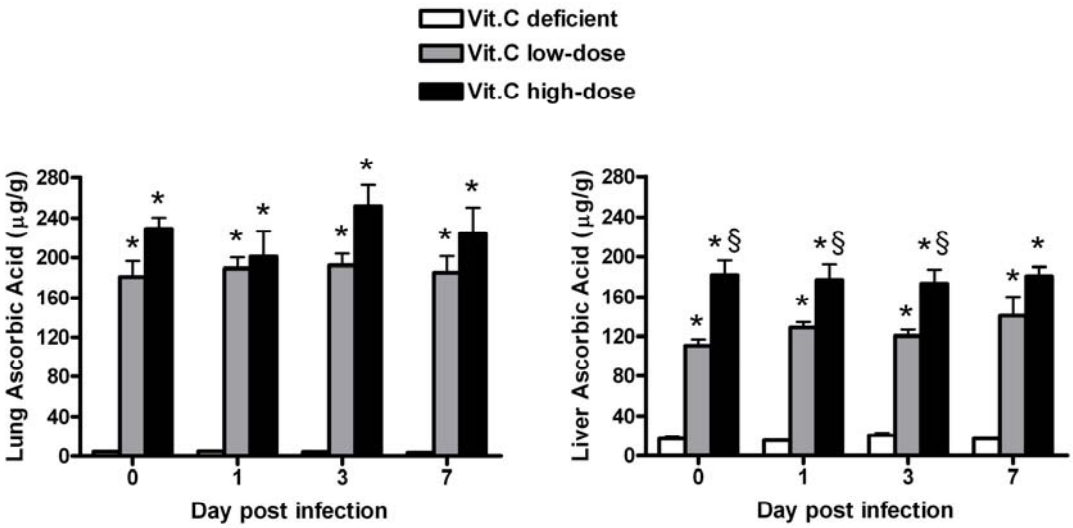


Figure 5.2: Lung pathology of gulo-/- mice prior to infection (day 0) and at 1, 3 and 7 days post-infection. *Gulo-/-* mice were provided with regular water, water containing 330 mg/L vitamin C or water containing 3.3 g/L vitamin C. Data are expressed as mean ± S.E. Asterisks indicates significant difference with low-dose vitamin C supplemented group. § indicate significant difference with the vitamin C deficient group.

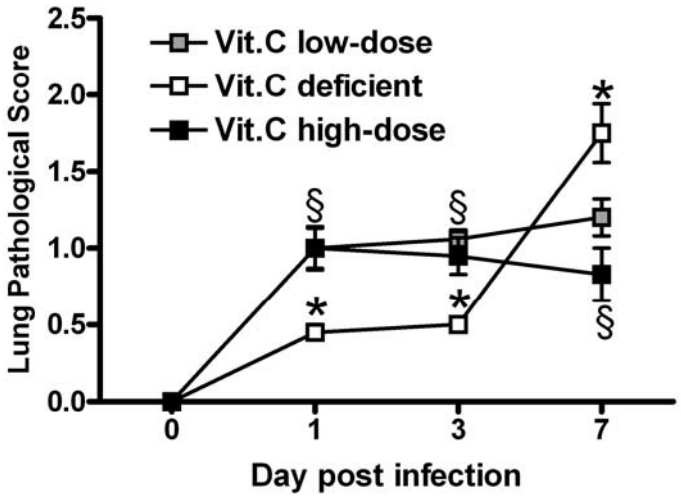


Figure 5.3: Influenza viral titers in the lung at 1, 3 and 7 days post-infection. *Gulo-/-* mice were provided with regular water, water containing 330 mg/L vitamin C or water containing 3.3 g/L vitamin C. Data are expressed as mean ± S.E.

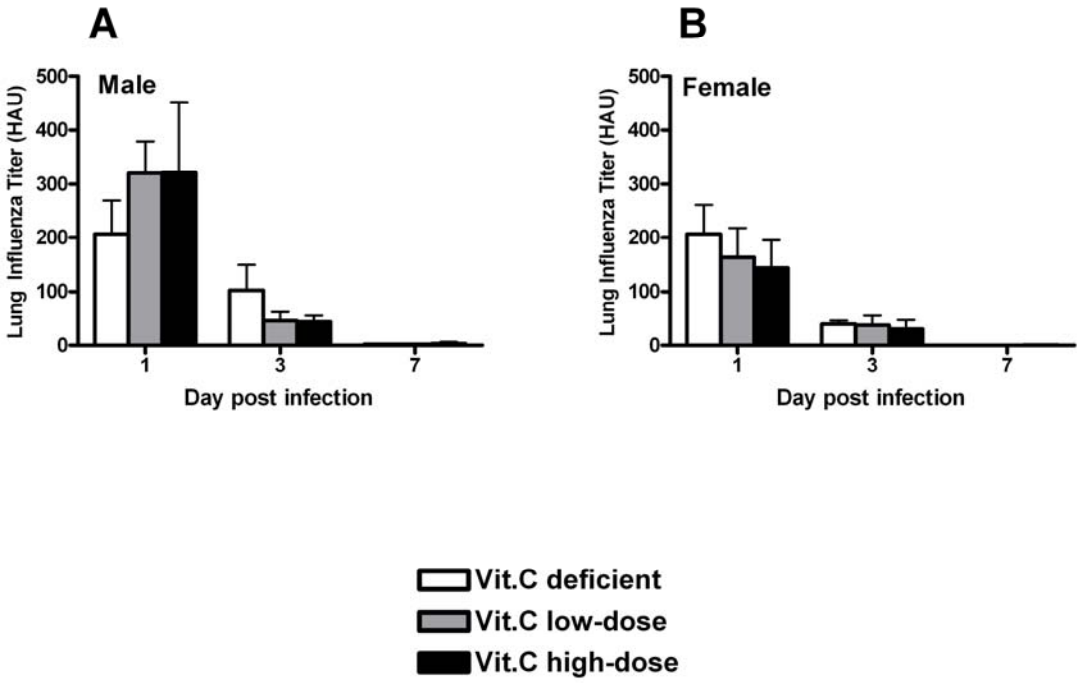


Figure 5.4: Lung mRNA levels for IFN-α and IFN-β from male gulo-/- mice prior to infection (day 0) and at 1, 3 and 7 days post-infection. *Gulo-/-* mice were provided with regular water, water containing 330 mg/L vitamin C or water containing 3.3 g/L vitamin C. Data are expressed as the ratio to day 0 low-dose vitamin C supplemented group ± S.E.

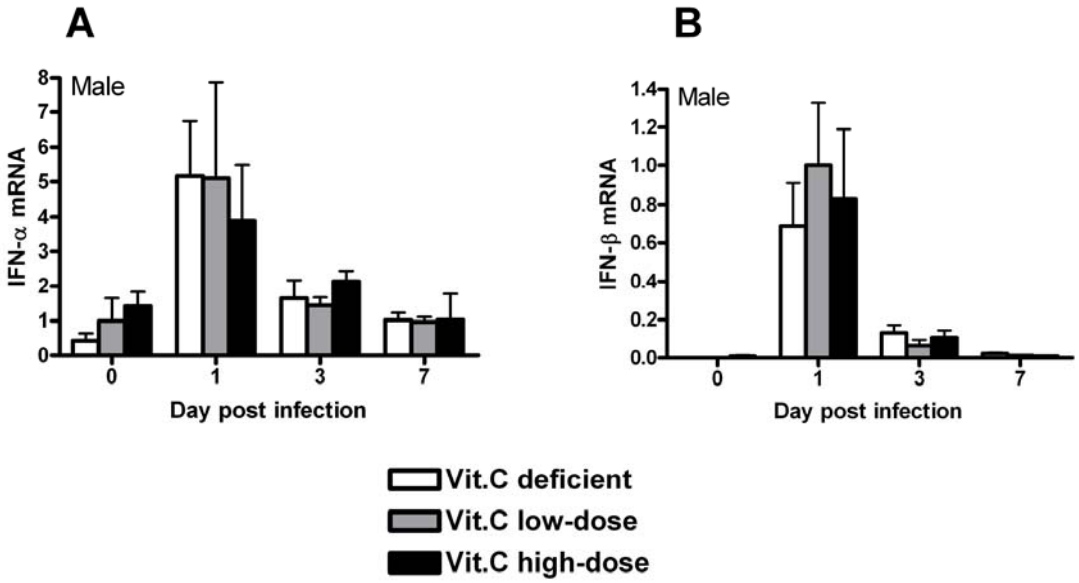


Figure 5.5: Lung mRNA levels for IFN-γ from gulo-/- mice prior to infection (day 0) and at 1, 3 and 7 days post-infection. *Gulo-/-* mice were provided with regular water, water containing 330 mg/L vitamin C or water containing 3.3 g/L vitamin C. Data are expressed as mean ± S.E. Asterisks indicate significant difference with the vitamin C deficient group ($P < 0.05$).

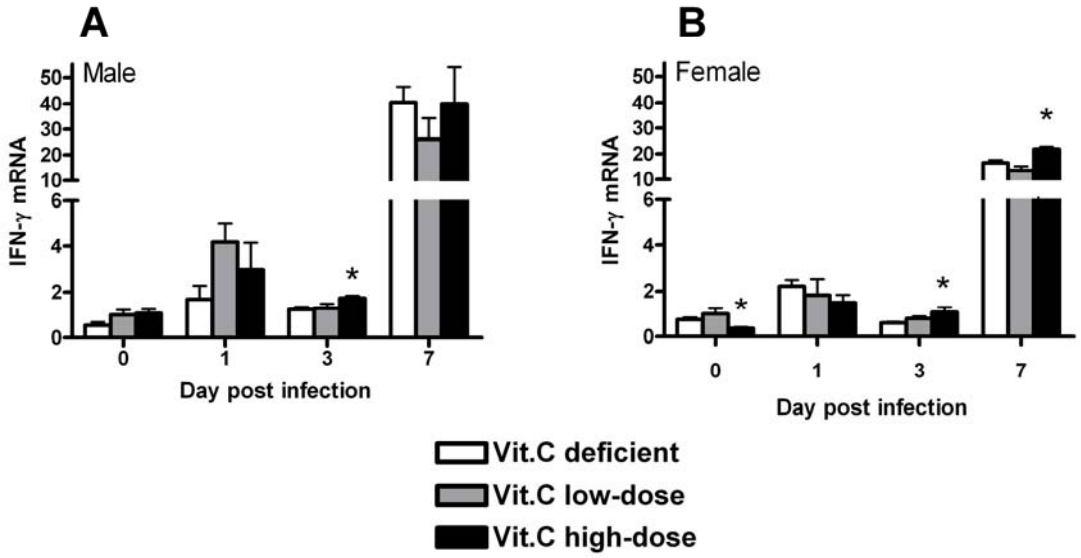


Figure 5.6: Lung mRNA levels for chemokines RANTES, MCP-1 and MIP-1α from gulo-/- mice prior to infection (day 0) and at 1, 3 and 7 days post-infection. *Gulo-/-* mice were provided with regular water, water containing 330 mg/L vitamin C or water containing 3.3 g/L vitamin C. Data are expressed as mean ± S.E. Asterisks indicate significant difference with the low dose supplemented group ($P < 0.05$). § indicate significant difference with the vitamin C deficient group ($P < 0.05$).

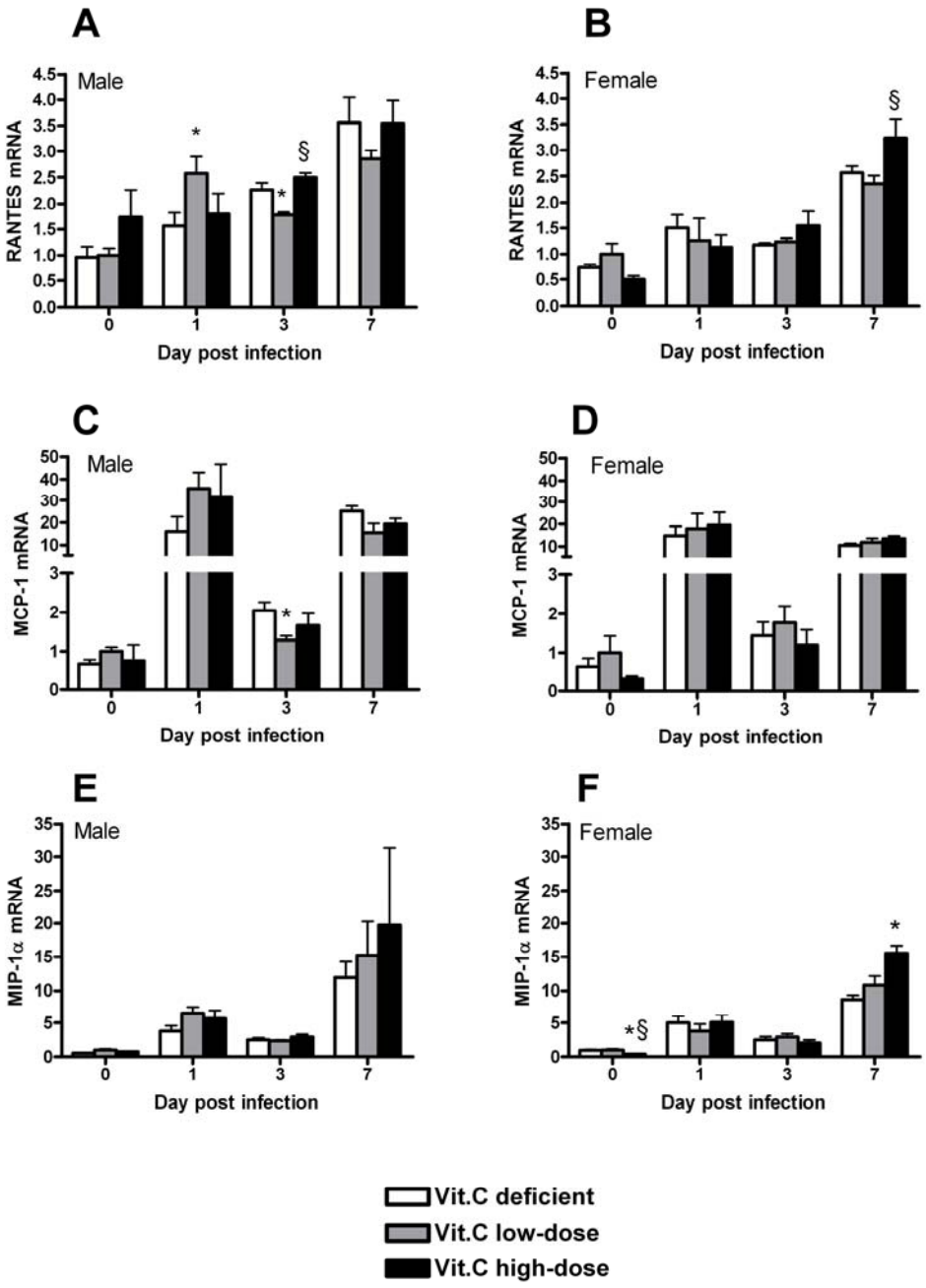

A
Male
RANTES mRNA
Day post infection
B
Female
RANTES mRNA
Day post infection
C
Male
MCP-1 mRNA
Day post infection
D
Female
MCP-1 mRNA
Day post infection
E
Male
MIP-1α mRNA
Day post infection
F
Female
MIP-1α mRNA
Day post infection
Vit.C deficient
Vit.C low-dose
Vit.C high-dose

Figure 5.7: Lung mRNA levels for cytokines IL-12, IL-1β, TNF-α and IL-6 from gulo-/- mice prior to infection (day 0) and at 1, 3 and 7 days post-infection. *Gulo-/-* mice were provided with regular water, water containing 330 mg/L vitamin C or water containing 3.3 g/L vitamin C. Data are expressed as mean ± S.E. Asterisks indicate significant difference with the low dose supplemented group ($P < 0.05$). § indicate significant difference with the vitamin C deficient group ($P < 0.05$).

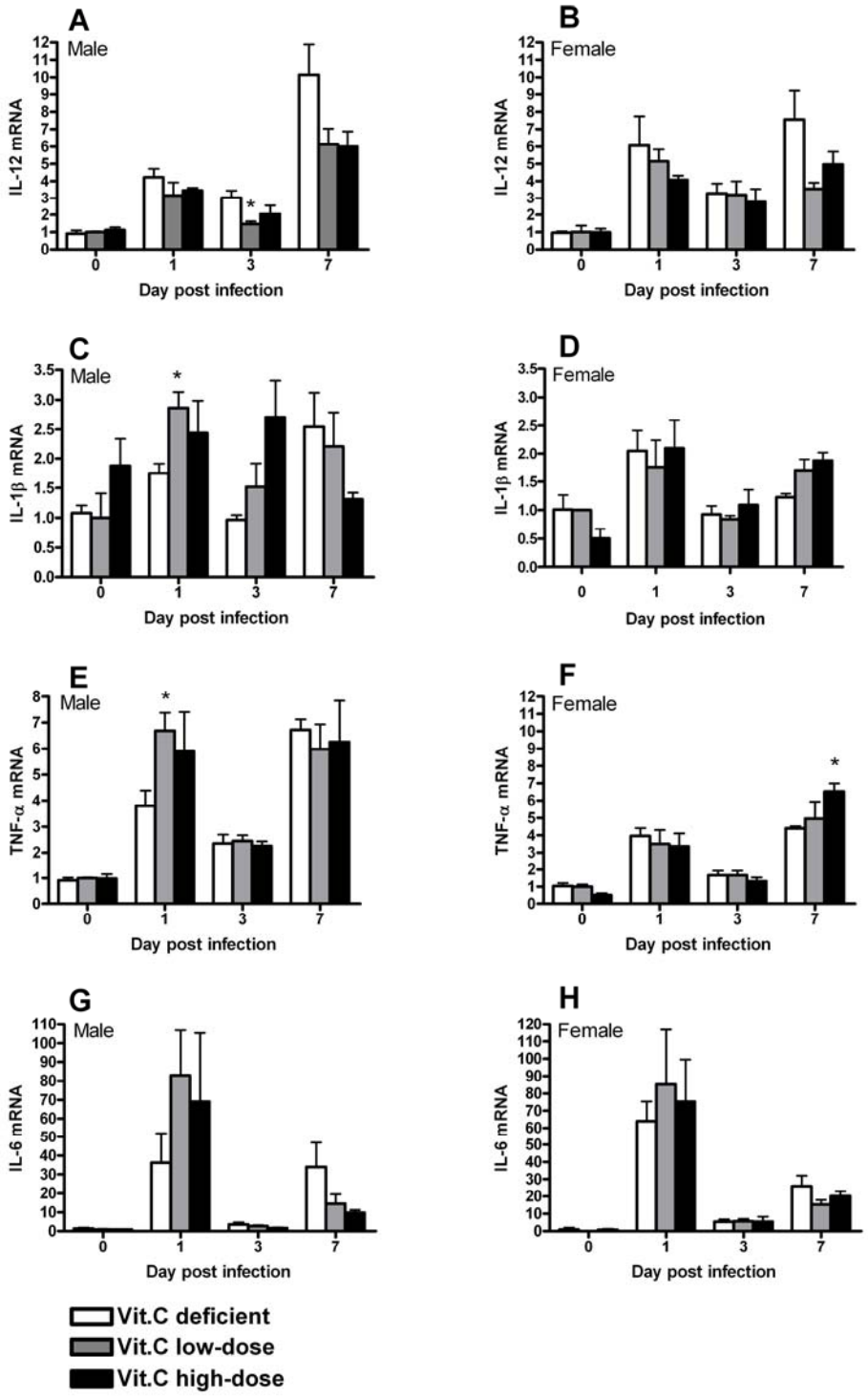

A
Male
IL-12 mRNA
Day post infection
B
Female
IL-12 mRNA
Day post infection
C
Male
IL-1β mRNA
Day post infection
D
Female
IL-1β mRNA
Day post infection
E
Male
TNF-α mRNA
Day post infection
F
Female
TNF-α mRNA
Day post infection
G
Male
IL-6 mRNA
Day post infection
H
Female
IL-6 mRNA
Day post infection
Vit.C deficient
Vit.C low-dose
Vit.C high-dose

Figure 5.8: Lung mRNA levels for IL-10 from male gulo-/- mice prior to infection (day 0) and at 1, 3 and 7 days post-infection. *Gulo-/-* mice were provided with regular water, water containing 330 mg/L vitamin C or water containing 3.3 g/L vitamin C. Data are expressed as mean ± S.E. Asterisks indicate significant difference with the low dose supplemented group ($P < 0.05$).

Figure 5.9: Vitamin C supplemented gulo-/- mice have a trend of decreasing NF-κB p65 nuclear translocation. ***Gulo-/-*** **mice were provided with regular water, water containing 330 mg/L vitamin C or water containing 3.3 g/L vitamin C. (A, B and C) p65 nuclear translocation were measured by Western blot. (E and F) Data were normalized to β-actin and expressed as mean ± S.E. § indicates significant difference with the vitamin C deficient group ($P < 0.05$).**

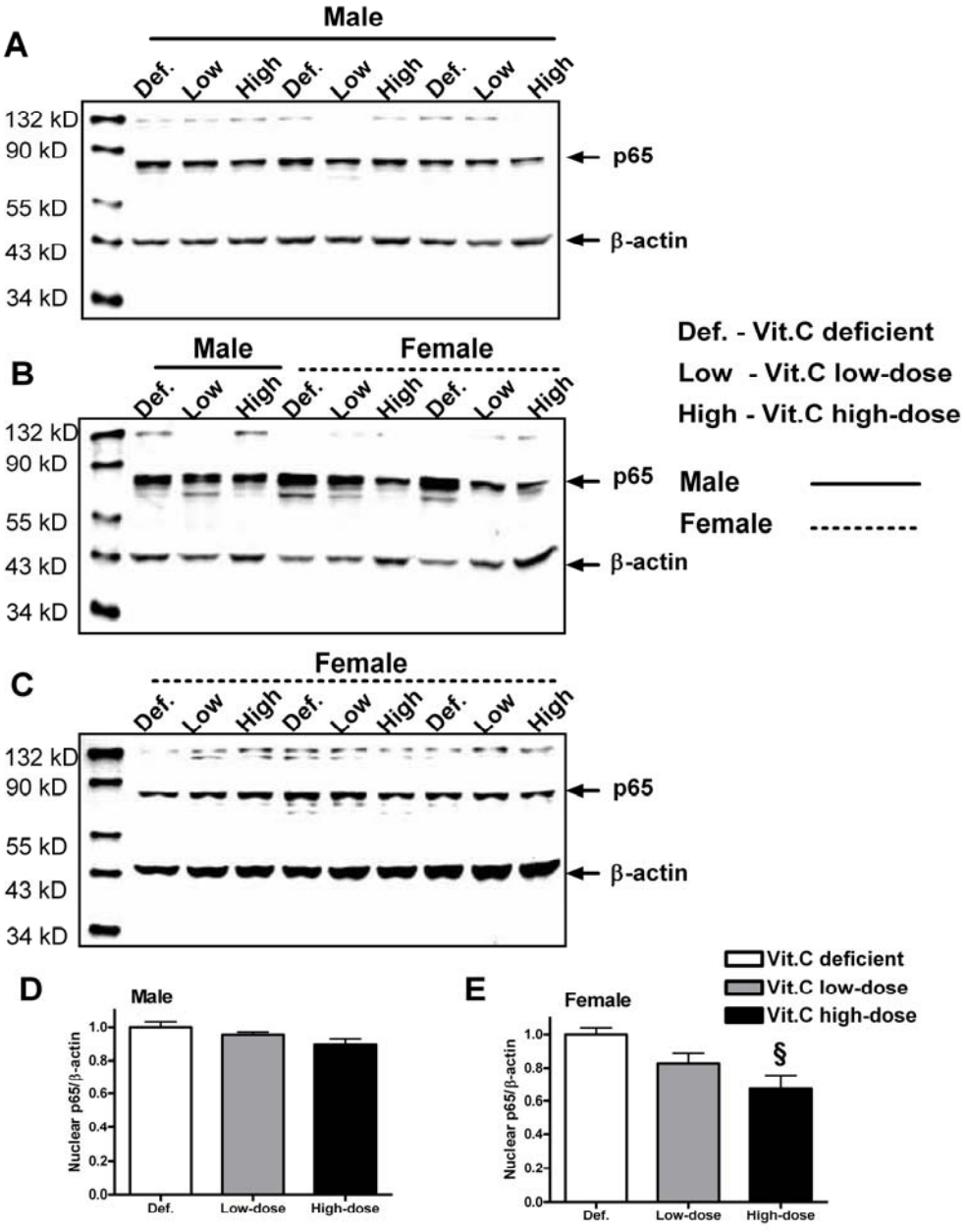

A
Male
Def. Low High Def. Low High Def. Low High
132 kD
90 kD
55 kD
43 kD
34 kD
p65
β-actin
B
Male
Female
Def. Low High Def. Low High Def. Low High
132 kD
90 kD
55 kD
43 kD
34 kD
p65
β-actin
Def. - Vit.C deficient
Low - Vit.C low-dose
High - Vit.C high-dose
Male
Female
C
Female
Def. Low High Def. Low High Def. Low High
132 kD
90 kD
55 kD
43 kD
34 kD
p65
β-actin
D
Male
Nuclear p65/β-actin
1.0
0.8
0.6
0.4
0.2
0.0
Def.
Low-dose
High-dose
E
Female
Nuclear p65/β-actin
1.0
0.8
0.6
0.4
0.2
0.0
§
Def.
Low-dose
High-dose
Vit.C deficient
Vit.C low-dose
Vit.C high-dose

Figure 5.10: Most p65 protein was located in the cytosol in the lung of male gulo-/- mice at day 1 post-infection. Mice were provided with regular water, water containing 330 mg/L vitamin C or water containing 3.3 g/L vitamin C. p65 protein was labeled with Alexa Fluor® 488 (Green). (A) Vitamin C deficient; (B) Low-dose vitamin C supplemented; (C) High-dose vitamin C supplemented.

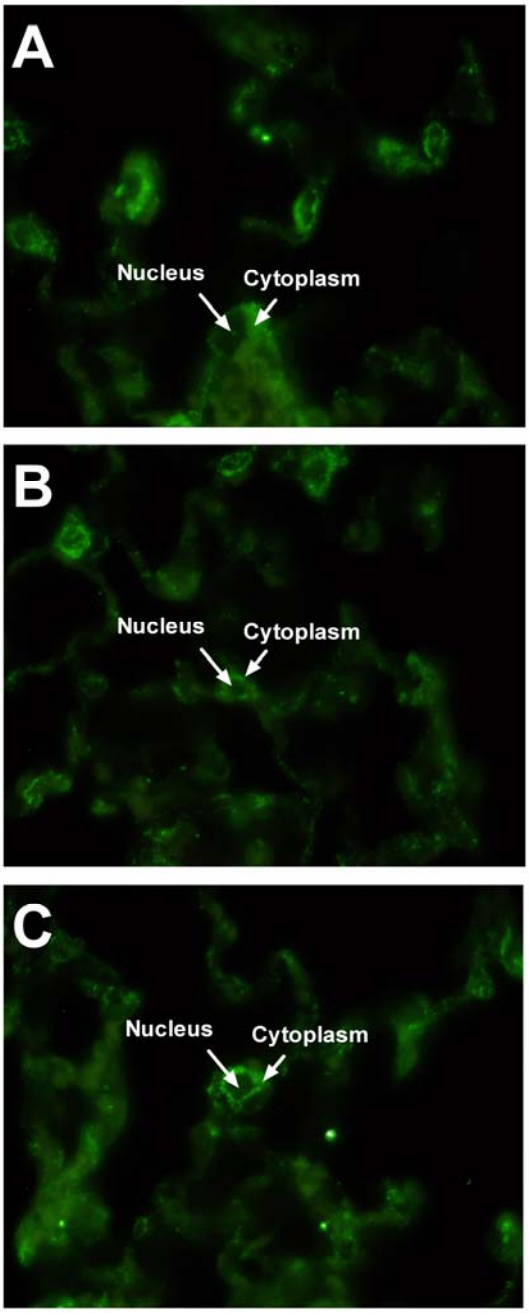
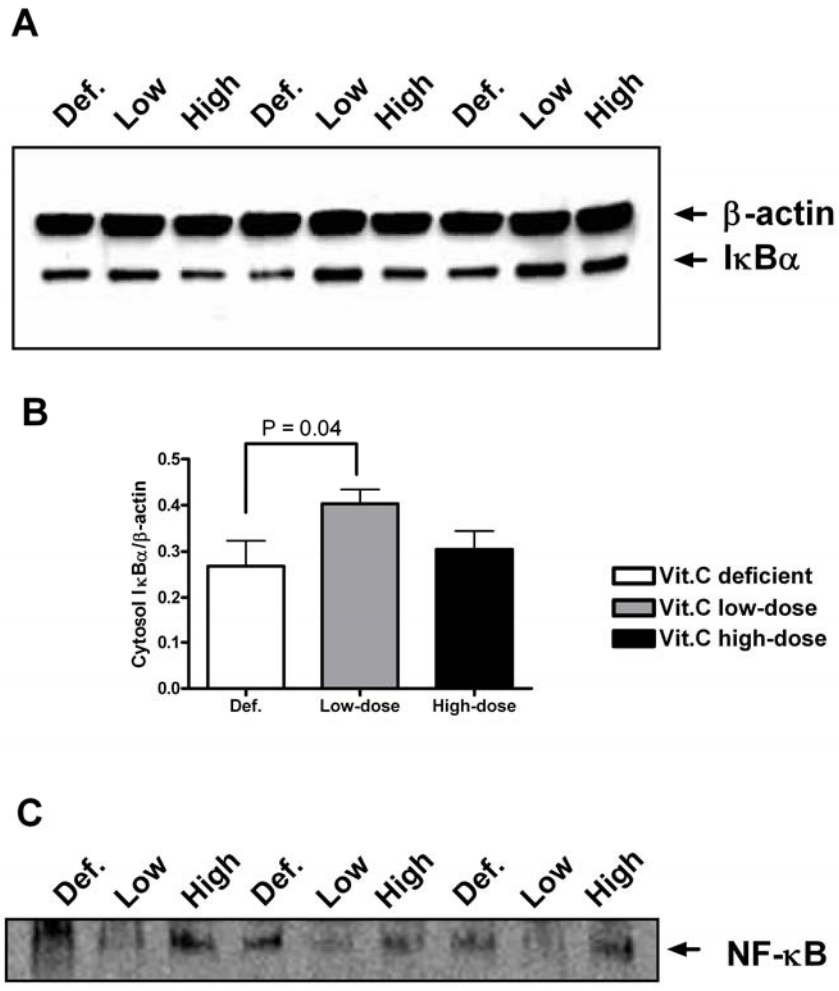


Figure 5.11: Vitamin C deficient male gulo-/- mice and high-dose vitamin C supplemented male gulo-/- mice had increased NF-κB activation in the lung at day 1 post-infection compared with low-dose vitamin C supplemented gulo-/- mice. (A) IκBα in the cytosolic fractions of the lung was measured by Western blot. (B) Densitometric analysis was conducted on immunoblots, and results are expressed as the ratio to β-actin ± S.E. (C) NF-κB DNA binding was measured in the nuclear fractions of the lung by EMSA.



Chapter VI

The effect of vitamin C supplementation and influenza virus infection on the activation of nuclear factor-κB (NF-κB)

A. ABSTRACT

This study was designed to investigate the effect of vitamin C supplementation on influenza infection-induced NF-κB activation. A549 cells and BEAS cells were supplemented with various concentrations of vitamin C for up to 4 days prior to infection with influenza A/Bangkok/1/79. At 8 hr post-infection, the amount of NF-κB p65 protein and the NF-κB activity in the nucleus of A549 cells correlated with the amount of influenza virus used for infection. Supplementation with 100 μM vitamin C down-regulated NF-κB nuclear translocation while supplementation with 500 μM vitamin C up-regulated NF-κB nuclear translocation in A549 cells both at baseline prior to influenza virus infection and at 8 hr post-infection. In contrast to A549 cells, BEAS cells had increased NF-κB nuclear translocation in response to 100 μM vitamin C and reduced NF-κB nuclear translocation in response to 500 μM vitamin C. These data suggest that the effect of vitamin C on NF-κB activation is concentration dependent and cell type specific.

B. INTRODUCTION

NF-κB is a dimeric eukaryotic transcription factor which is activated by a variety of inflammatory stimuli, including cytokines and viral infection [73, 75]. In unstimulated cells, the inactive form of NF-κB is retained in the cytosol by associating with inhibitory factors known as IκB family of inhibitory proteins. Upon stimulation, IκBs are rapidly phosphorylated and degraded via proteasomal pathways. The degradation of IκBs release NF-κB, allowing transport to the nucleus where they bind to DNA and regulate gene transcription. The Rel/NFκB family of transcriptional factors regulate expression of numerous cellular and viral genes and play important roles in immune and stress responses, inflammation, and apoptosis [71-74].

Infection with influenza virus activates NF-κB by several mechanisms. Mammalian toll-like receptor 3 (TLR3) recognizes the double-stranded RNA (dsRNA) that is produced during influenza virus replication and activates NF-κB through MAPK p38, PI3K/Akt signaling [17, 18]. dsRNA also activates protein kinase R (PKR) by autophosphorylation. Phosphorylated PKR activates NF-κB by phosphorylating IκBs [16]. In addition to the dsRNA mechanisms, NF-κB is activated by the accumulation of influenza viral proteins in the endoplasmic reticulum membrane, which causes an "ER overload" and calcium release from ER. This mechanism involves the generation of free radicals (reactive oxygen intermediates; ROIs) and activation of IKK-β [250, 251].

NF-κB is activated by oxidative stress through the increased phosphorylation of MAPK p38 and Akt [69, 70]**.** As mentioned previously, both MAPK p38 and Akt are intermediates in the signaling from TLR3-dsRNA recognition to NF-κB activation and ROIs are also involved in the "ER overload" mechanism of NF-κB activation, suggesting that

oxidative stress may be able to amplify the effects of dsRNA and "ER overload" on NF-κB activation in a synergistic manner.

As an antioxidant vitamin, vitamin C suppresses TNF-α induced NF-κB activation by inhibiting IκBα phosphorylation [170, 247]. In the current study, we investigated the effect of vitamin C on influenza infection-induced NF-κB activation in tissue culture.

C. MATERIALS AND METHODS

Cell culture and treatment. The BEAS-2B cell line was derived by transforming human bronchial cells with an adenovirus 12-simian virus 40 construct [252]. BEAS-2B cells were grown to 90-100% confluence on tissue culture-treated Costar 6- or 12-well plates in keratinocyte basal medium (KBM) supplemented with 30 μg/ml bovine pituitary extract, 5 ng/ml human EGF, 500 ng/ml hydrocortisone, 0.1 mM ethanolamine, 0.1 mM phosphoethanolamine, and 5 ng/ml insulin, as described previously [253, 254]. A549 (American Type Culture, Rockville, MD) is a human pulmonary type II epithelial cell-like line derived from a patient with alveolar cell carcinoma of the lung. A549 cells were cultured in F-12K media with 10% fetal bovine serum (FBS, GIBCO-BRL, Gaithersburg, MD) and 1% penicillin and streptomycin (GIBCO-BRL). For experiments, tissue culture media were supplemented with various concentrations of vitamin C for 4 days and the media was changed daily.

Influenza infection. Influenza A/Bangkok/1/79 was propagated in 10-day-old embryonated hen's eggs [210]. The virus-containing allantoic fluid was collected and stored at -80°C. For infection of cells, 1, 2 or 3 M.O.I. (multiplicity of infection) of influenza virus was used.

Separation of cytoplasmic and nuclear fractions. After washing cells with ice-cold phosphate-buffered saline, 200 μl of cold cytoplasmic extraction buffer (CEB) (10 mM Tris-HCl, pH 7.9, 60 mM KCl, 1 mM EDTA, 1 mM DTT) with protease inhibitors (1 mM AEBSF, 0.8 μM aprotinin, 50 μM bestatin, 15 μM E-64, 20 μM leupeptin, 10 μM pepstatin A; all part of Protease Inhibitor Cocktail Set III, Calbiochem) was added to each well. Cells were scraped off and transferred to a microcentrifuge tube. The cells were held on ice for 15 min,

then NP-40 (Sigma) was added to a final concentration of 0.1% and the tube vortexed. Nuclei were pelleted by centrifugation at 15,000 × g for 30 s. The supernatant, containing the cytoplasmic fraction, was mixed with one-quarter volume of 4× loading buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.7 M β-mercaptoethanol, 0.05% bromophenol blue), denatured at 95°C for 10 min, and stored at -70°C for immunoblot analysis. Protein content of a small aliquot of the cytoplasmic fraction was determined using the DC Bradford assay (BioRad, Richmond, CA). The nuclei were washed with CEB/ protease inhibitors (PI) and centrifuged again at 15,000 × g for 30 s. The supernatant was aspirated and the nuclei were incubated for 10 min on ice in nuclear extraction buffer (20 mM Tris-HCl, pH 8.0, 400 mM NaCl, 1.5 mM Mg Cl_2, 1.5 mM EDTA, 1 mM DTT, 25% glycerol) with PI. After brief centrifugation, the supernatants, containing the nuclear fraction, were either stored at −80°C until analysis by electrophoretic mobility shift assay or denatured and stored for immunoblot analysis.

Western blot. Cytoplasmic and nuclear fractions were separated by 12% (wt/vol) sodium dodecyl sulfate (SDS) polyacrylamide gels and electrophoretically blotted onto Immobilon-P membranes (Millipore) according to standard procedures. The membranes were blocked with 5% non-fat milk and treated with rabbit anti-IκBα (9242, Cell Signaling Technology, Danvers, MA) or anti-NF-κBp65 (sc-109, Santa Cruz Biotechnology, Santa Cruz, CA) antibodies overnight at 4°C. Membranes were then incubated with a secondary goat anti-rabbit IgG conjugated with horseradish peroxidase (7074, Cell Signaling Technology, Danvers, MA) for 1h at room temperature and stained with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). The chemiluminescent signals were acquired using a 16-bit CCD camera (GeneGnome system; Syngene, Frederick, MD).

EMSA. EMSA was conducted as previously described [255]. Briefly, an oligonucleotide probe (sequence from 5' to 3': GGCTGGGGATTCCCCATCT) for NF-κB binding site on myosin heavy chain class II gene was labeled by incubating 15 U T4 polynucleotide kinase (New England Biolabs, Beverly, MA), 100 ng double-stranded probe, and 100 μCi [γ-^{32}P]ATP at 37°C for 30 min. Unincorporated ^{32}P was removed using a desalting column (Nuc Trap, Stratagene, San Diego, CA), and DNA-protein binding reactions were performed for 10 min at room temperature in a mixture containing 2 μg nuclear extract, 1 μl labeled probe, 10 μl running buffer (10 mM Tris · HCl, pH 7.5, 50 mM NaCl, 2 mM EDTA, 1 mM DTT, and 5% glycerol), and 2 μg poly(dI-dC) (Roche Molecular Biochemicals). Samples were separated by electrophoresis through a 4.5% nondenaturing polyacrylamide gel containing 0.5× Tris-borate-EDTA. Gels were dried, and radiolabeled species were autoradiographed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Promoter-reporter assay. A κB-dependent promoter-reporter construct, pNF-κB-luc (Stratagene), was used. It was composed of a 5× tandem repeat of the NF-κB response element of the mouse Igκ gene intronic enhancer cloned upstream of a TATA box and a firefly luciferase cDNA. A constitutively active SV-40 promoter-β-galactosidase construct, pSV-β-galactosidase (Promega, Madison, WI), was used to adjust for well-to-well variation in cell number and transfection efficiency. A549 cells grown to 60 to 80% confluence in 24-well tissue culture dishes were cotransfected with 250 ng of the pNF-κB-luc and 25 ng of pSV-β-galactosidase using 1.5 μg of DOTAP transfection reagent (Boehringer Mannheim). Forty-eight hours after transfection, cultures were infected with 1, 2 or 3 M.O.I. of influenza virus or treated with TNF-α as a positive control. At 16 hr post-infection, Luciferase and β-

galactosidase activity was determined using the Dual Light reporter gene assay system (Perkin-Elmer) and an AutoLumat LB953 luminometer (Berthold Analytical Instruments, Nashua, NH). Promoter activity was estimated as specific luciferase activity (luciferase counts per unit β-galactosidase counts) and expressed as fold induction over the respective media control.

Statistics. The promoter-reporter assay dada were analyzed by one-way ANOVA.

D. RESULTS

The NF-κB activation in A549 cells correlates with the dose of influenza virus used for infection. As the amount of infecting virus increased, there was an increase in the amount of p65 protein in the nucleus (figure 6.1 A), an increase in NF-κB DNA binding activity in the nucleus (figure 6.1 B) and an increase in NF-κB activated gene transcription as measured by a promoter-reporter assay (figure 6.1 C).

With 100 μM vitamin C supplementation, A549 cells had slightly less NF-κB DNA binding activity in the nucleus prior to infleuza infection and at 8 hrs post-infection compared with the unsupplemented controls. In contrast, the 500 μM vitamin C supplementation caused an up-regulation of NF-κB DNA binding in the nucleus prior to and post infection compared with the unsupplemented controls (figure 6.2).

When the effect of vitamin C supplementation on NF-κB activation was measured in BEAS cells by Western blot, the 100 μM vitamin C supplementation down-regulated IκBα in the cytoplasm and up-regulated p65 in the nucleus. In contrast, the 500 μM vitamin C supplementation appeared to up-regulate IκBα in the cytoplasm and down-regulate p65 in the nucleus, especially post influenza virus infection (figure 6.3).

E. DISCUSSION

Influenza virus infection activates NF-κB, a transcription factor that regulates the expression of numerous genes involved in the inflammatory response. NF-κB is also a redox sensitive transcription factor that is activated by oxidative stress [70]. In the current study, we investigated the effect of vitamin C supplementation on NF-κB activation in A549 cells and BEAS cells.

In A549 cells, the 100 μM vitamin C supplementation appeared to suppress NF-κB activation at baseline prior to influenza infection and have little effect post-infection compared with the unsupplemented controls. Surprisingly, the 500 μM vitamin C supplementation stimulated NF-κB activation both at baseline (prior to infection) and post-infection, suggesting vitamin C may act as a prooxidant at this concentration. In contrast, the effect of vitamin C supplementation on NF-κB activation in BEAS cells was opposite to the results in A549 cells. The 100 μM vitamin C supplementation up-regulated NF-κB activation while the 500 μM vitamin C supplementation appeared to have some inhibitory effect on NF-κB activation both before and after influenza infection.

There has been controversy regarding the form of vitamin C, reduced or oxidized, transported into cells. Vitamin C is transported as ascorbic acid in specialized cells by sodium-dependent ascorbic acid transporters SVCT-1 and -2 [256]. However, current evidence indicates that dehydroascorbic acid, not ascorbic acid, is preferentially transported into cells and the transportation is mediated by glucose transporters [257-268]. All cells have glucose transporters and take up dehydroascorbic acid that is reduced by the glutathione system and trapped intracellularly as ascorbic acid [262], while only specialized cells such as the specialized tissues in the brain and eye, directly transport ascorbic acid using the sodium-

dependent transporters [256, 269]. It has also been demonstrated that vitamin C has prooxidant properties, especially in the presence of transition metals [138]. Therefore, if ascorbic acid is added to tissue culture media and is not transported into the cells immediately, it will have a chance to react with the transition metal components of the media or transition metals released from dead cells, thereby enhancing the oxidative stress status of the cells.

It is possible that A549 cells had a low ability to transport vitamin C as ascorbic acid, whereas BEAS cells may be able to transport ascorbic acid efficiently by the sodium-dependent transporters. When a high concentration of ascorbic acid was added, it acted as a prooxidant extracellularly in the A549 cell culture but as an antioxidant intracellularly in the BEAS cell culture. In contrast, when a relatively low concentration of ascorbic acid was added to BEAS cells, an optimal intracellular ascorbic acid concentration was not achieved and the prooxidative effect of extracellular ascorbic acid was dominant, providing a possible explanation for the activation of NF-κB by 100 μM ascorbic acid in BEAS cells.

Thus, the antioxidative/prooxidative effect of vitamin C supplementation *in vitro* is likely determined by a balance between the extracellular ascorbic acid as a potential prooxidant and the intracellular ascorbic acid as an antioxidant, as well as by the ability of cells to transport vitamin C as ascorbic acid using the sodium-dependent transporters. The difference in the effect of vitamin C supplementation between A549 cells and BEAS cells may be explained by differences in the efficiency of ascorbic acid transportation, the transition metal content in the media, and the sensitivity to oxidative stress between the two cell types.

However, because the oxidative stress markers such as glutathione levels were not measured in the current study, therefore we cannot rule out the possibility that vitamin C up-regulated NF-κB signaling by mechanisms other than its antioxidant properties.

In summary, this study demonstrated that vitamin C supplementation *in vitro* may down-regulate or up-regulate NF-κB activation with or without influenza infection, depending on the vitamin C concentration and the cell type. Further studies investigating the factors that affect the antioxidant/prooxidant properties of vitamin C are warranted.

Figure 6.1: Influenza virus infection-stimulated NF-κB activation in A549 cells. (A) The amount of p65 protein in the nuclear fractions was determined by Western blot. The doses of influenza virus used for infection are indicated. TNF-α stimulated cells were used as a positive control. (B) The NF-κB DNA binding activity in the nuclear fractions were determined by EMSA assay. The doses of influenza virus used for infection are indicated. (C) The NF-κB activation of gene transcription was determined by a promoter reporter assay. The doses of influenza virus used for infection are indicated. RLU – Relative light units. Asterisks indicate significant difference with control. § indicate significant difference with the M.O.I = 1 group ($P < 0.05$).

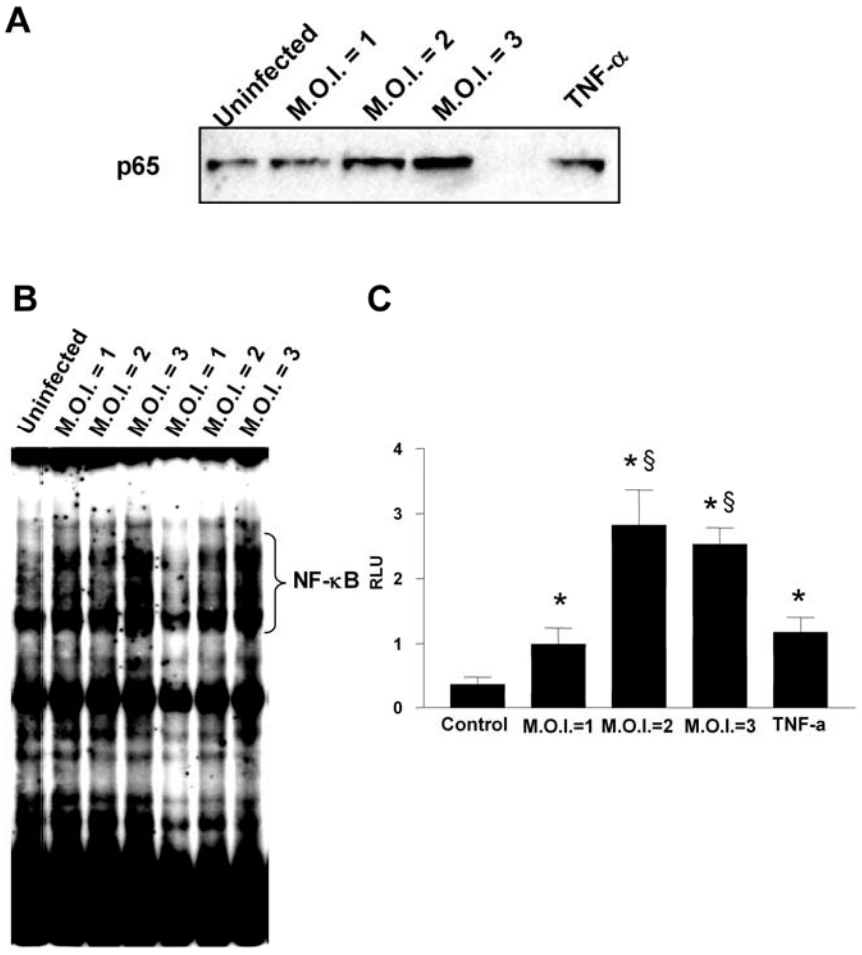


Figure 6.2 The effect of vitamin C supplementation and influenza infection on NF-κB DNA binding in A549 cells. The concentrations of vitamin C in the media are indicated. Sample 4, 5 and 6 were from influenza virus infected A549 cells (M.O.I. = 2).

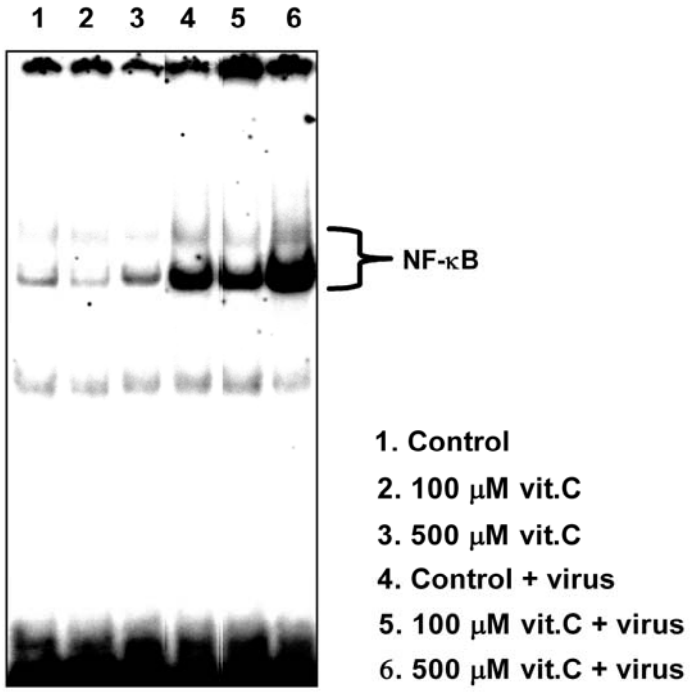
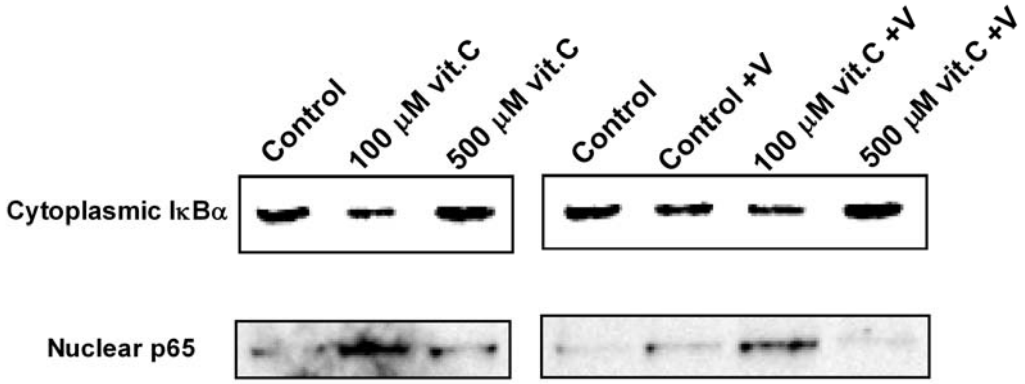


Figure 6.3: The effect of vitamin C supplementation and influenza infection on the cellular levels of IκBα and p65 in BEAS cells. The IκBα in the cytoplasmic fractions and the p65 in the nuclear fractions were determined by Western blot. The concentrations of vitamin C in the media are indicated. "+V" indicates the sample was from cells infected with influenza virus (M.O.I. = 2).



Chapter VII

Summary and Concluding Remarks

My graduate research has focused on the effect of nutritionally-induced oxidative stress on the immune response to influenza virus infection. Two nutrients were studied: selenium (Se) and vitamin C. Both Se deficiency and vitamin C deficiency induce oxidative stress. For my research, the effect of Se deficiency was studied using a Se-deficient mouse model. The studies on the influence of vitamin C on viral infection utilized *in vitro* tissue culture models supplemented with vitamin C and an *in vivo* model using *gulo-/-* knockout mice that can not synthesize vitamin C.

A. Se deficiency and influenza A/Puerto Rico/8/34 infection

Previous studies in our lab have demonstrated that Se-deficient mice infected with a mild human strain of influenza virus, influenza A/Bangkok/1/79, developed much more severe lung pathology compared with Se-adequate mice, the immune response was altered in Se-deficient mice, and importantly, mutations occurred in the viral genome allowing the virus to aquire a more virulent genotype [204, 209].

The first part of my graduate research looked at whether Se deficiency would have a similar effect if a virulent strain of influenza is used for infection instead of a mild strain. Influenza A/PR8/34 (PR8) is a mouse-adapted strain of influenza virus which replicates efficiently in mice [208] and induces a severe inflammatory response in the infected lung [45]. Surprisingly, when infected with influenza PR8, Se-deficient mice had a lower mortality rate (0%) at day 7 post-infection compared with Se-adequate mice (50%). When the virus isolated from the infected mice was sequenced and compared with the sequence of the virus used for infection, no genome mutations were detected in virus isolated from either Se-adequate mice or Se-deficient mice.

Although there were no mutations in virus genome, Se deficiency did induce significant changes in the immune response. Infection with influenza virus typically induces a Th1-dominant immune response. However, the immune response to influenza in Se-deficient mice was skewed toward a Th2 type when compared with Se-adequate mice. Glutathione (GSH) levels in the immune cells play an important role in determining the Th1/Th2 balance [223, 224]. Se-deficiency may tip the Th1/Th2 balance by affecting the GSH level in the immune cells.Thus, the role of Se in altering immune function and the subsequent ability to handle a viral infection most likely is due to its anti-oxidant properties.

B. The effect of vitamin C on influenza virus infection

Because my work with Se suggested that a deficiency in Se leads to increased oxidative stress resulting in impaired immune function, I wanted to determine if other antioxidants would have a similar effect.

In addition to serving as a cofactor for several enzymes involved in important metabolic pathways, vitamin C is an important water-soluble antioxidant vitamin. The effect of vitamin C on common colds was made famous by Linus Pauling and has been an on-going debate for decades. The beneficial effect of vitamin C on the prevention or treatment of common colds has been supported by some studies [245, 270, 271] while questioned by others [228]. More than 200 viruses are able to cause common colds or “flu” symptoms. However, very few vitamin C supplementation studies have focused specifically on the influenza virus.

The vitamin C part of my graduate research started with studying the effect of vitamin C supplementation on influenza-induced NF-κB activation *in vitro*. This study demonstrated that the effect of vitamin C on NF-κB activation was concentration dependent and cell type

specific. Vitamin C down-regulated NF-κB activation or up-regulated NF-κB activation depending on the concentration of vitamin C. It has been suggested that vitamin C has prooxidant properties *in vitro* [138] due to hydroxyl radical-forming Fenton reaction with transition metals. In addition, transport of vitamin C as ascorbic acid into tissue culture is poor [248]. Therefore, the buildup of vitamin C outside of the cells may result in increased possibility for oxidant formation, and subsequent oxidative stress.

Before the creation of the *gulo-/-* mice, it was impossible to study vitamin C deficiency in mice because, unlike humans, mice are able to synthesize vitamin C in the liver. Vitamin C supplementation studies in mice were also questionable due to uncontrolled *de novo* vitamin C synthesis. The *gulo-/-* knockout mice were originally created in Maeda's lab at UNC to study the effect of vitamin C deficiency on vascular diseases [171]. Due to the *in vitro* observations we had made in cell cultures, we wondered how vitamin C deficiency/supplementation would affect the immune response to influenza infection *in vivo*. The creation of these mice provided us an opportunity to continue to further study the effects of vitamin C.

A supplementation of 330 mg/L vitamin C in the drinking water maintains the plasma ascorbic acid levels in *gulo-/-* mice at a concentration 60% of wild-type mice and is adequate for normal physiological functions. In our study, when vitamin C deficient *gulo-/-* mice and vitamin C adequate *gulo-/-* mice were infected with a mild strain of influenza virus (influenza A/Bangkok/1/79), vitamin C deficient mice had a delayed immune response and greater lung pathology late post infection at day 7, suggesting an adequate dietary vitamin C intake is essential for a normal immune response to influenza infection. The effect of vitamin C

deficiency also had a clear sex difference, with the changes in the chemokine and cytokine expression only seen in male mice.

Based on these observations, we hypothesized that the eradication of a virulent strain of influenza virus may be impaired in vitamin C deficient mice and tested our hypothesis. Indeed, when the mouse-adapted influenza PR8 was used for infection, vitamin C deficient *gulo-/-* male mice had a significantly higher lung viral titer at day 7 post infection compared with vitamin C adequate *gulo-/-* male mice. However, the viral titers was not different in female *gulo-/-* mice, again demonstrating a clear sex difference with respect to vitamin C's effects.

We then tested whether a 10× higher dose vitamin C supplementation (3.3 g/L) would further decrease the influenza induced lung pathology and optimize the immune response. The high-dose vitamin C supplementation appeared to have dual-effects. Similar to the low-dose supplementation, the high-dose supplementation also effectively limited lung pathology late post-infection. However, in contrast to the low-dose supplementation, the high-dose supplementation did not down-regulate influenza induced NF-κB activation. The high-dose vitamin C supplementation also enhanced the Th1 type response at day 3 and 7 compared with the low-dose supplementation or vitamin C deficiency.

C. Concluding Remarks

In conclusion, data obtained from Se-deficient mice and vitamin C deficient/supplemented *gulo-/-* mice demonstrate the immune response to influenza infection is deeply affected by the oxidative stress of the host. Antioxidant nutrients play an important role in maintaining oxidative balance. Once that balance is tipped in favor of pro-oxidation (e.g. due to reduced antioxidant intake), the immune system and subsequent control of viral

infections is impaired. The Se/influenza PR8 study added new information to the "Se and influenza infection" topic that our lab has long been interested in. The unexpected beneficial effect of Se deficiency in protecting the mice from influenza induced mortality suggests that Se deficiency can have totally different effects when the mice are infected with different strains of virus. Therefore, we should always be careful when trying to extrapolate the results of one study to others. For our studies with influenza infection in *gulo-/-* mice, they demonstrate that an adequate vitamin C intake is essential for a normal immune response and supports the observations by other researchers that vitamin C supplementation helps relieve flu symptoms. Both of our *in vitro* and *in vivo* studies demonstrate that low-dose and high-dose vitamin C supplementation have different effects on the influenza induced inflammatory response. This is the first time that *gulo-/-* knockout mice were used to study the effect of vitamin C on infectious diseases. Further studies with other doses of vitamin C supplementation and studies that will investigate the mechanisms responsible for this "dose difference" and sex difference are warranted.

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