Exploring the effects of dietary zinc deficiency on

T-cell maturation and function in the growing rat

by

Heather Joy Hosea

A thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfillment of the requirements of the degree of

Doctor of Philosophy

Inter-Departmental PhD program in Food and Nutritional Sciences

University of Manitoba

Winnipeg

Date of Defense: December 15, 2006

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

*****

COPYRIGHT PERMISSION

Exploring the effects of dietary zinc deficiency on T-cell maturation and function in the growing rat

by

Heather Joy Hosea

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

of

Doctor of Philosophy

ABSTRACT

The objectives of this thesis were to characterize the immunological effects of dietary zinc deficiency in a growing rat model, investigate the time course for recovery of lymphoid tissue, and explore potential mechanisms behind the immunodeficiency including increased lymphocyte susceptibility to apoptosis via differential expression of the zinc finger signaling protein $p56^{lck}$ and/or altered hypothalamus-pituitary-adrenal stress axis activity. Zinc-deficient rats (ZD) become anorexic; therefore, a diet-restricted group (DR) was included to separate the effects of zinc deficiency from malnutrition.

Providing zinc to ZD resulted in complete recovery of T-cell numbers and lymphoid organ weight before body weight, indicating a priority for recovery of lymphoid organs. Compared to the control group (CTL), ZD had higher thymocyte $p56^{lck}$ protein levels, which could theoretically alter thymocyte maturation or increase apoptosis. ZD had a higher proportion of thymic cytotoxic T-cells compared to CTL, but there was no effect of diet on apoptosis. ZD and DR had higher serum corticosterone concentrations compared to CTL, but the increased corticosterone concentrations were not associated with increased apoptosis or a decreased production of Th1 cytokines.

ZD had fewer recent thymic emigrants ($CD90^{+}TCR\alpha\beta^{+}$) compared to both DR and CTL. There was no difference in the proportion of $CD90^{+}$ thymocytes or the first two stages of post-thymic development based on CD45RC, RT6.1, and CD90 cell surface labeling of splenic T-cells. However, ZD, DR and marginally zinc deficient rats had a 42% lower proportion of late thymic emigrants

($TCR\alpha\beta^+CD90^+CD45RC^+RT6.1^+$) compared to CTL. The lower proportion of $CD90^+$ T-cells in ZD does not appear to be due to a defect in thymic production or increased apoptosis in the spleen, but future studies should examine the kinetics of thymic output/post-thymic maturation and include other organs to determine if the late thymic emigrants have homed to another organ.

This thesis has characterized the effects of dietary zinc deficiency on the T-cell populations of growing rats and provides a novel mechanism to explain the immunodeficiency associated with dietary zinc deficiency: a lower proportion of recent thymic emigrants over time will limit the T-cell repertoire, leaving the subject more susceptible to infection.

ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. Carla Taylor for her time, advice, and freedom to explore ideas and opportunities within and beyond my thesis work that have allowed me to travel further down the road towards becoming an independent researcher than any other advisor would normally allow.

I would like to thank Dr. Edward Rector for his invaluable help with the flow cytometry portion of my project; it wouldn't and couldn't have happened with out him. The missed dinners with his family because I had samples to run, and even crawling in to the lab to run my samples while suffering from pneumonia is far beyond the call of duty and it can not be put into words how grateful I am for his dedication. Besides the technical assistance and guidance provided by Dr. Rector, I would also like to thank him for being a mentor and the hours spent discussing research and life in academia are worth more than their weight in gold. I am honored to have had the opportunity to be his "apprentice".

I would like to thank the other members of my advisory committee Dr. Harold Aukema, Dr. Donna Chow, and Dr. Hope Weiler for their guidance, and being there when I truly needed them. They urged me to become the "Queen" of something, and I hope I have come close to achieving that ideal.

I could not have achieved even a tenth of what I have without the unwavering support of my family and friends. There are countless examples of how each and everyone of them have cleared the path to where I am today, but I think a good example would be how my grandmother told me as a young girl that I can do much more than bring a man a cup of coffee.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACTH	adrenocorticotropic hormone
ANOVA	analysis of variance
ConA	concanvalin A
CTL	control group
DAPI	4',6-diamidino-2-phenylindole
DEX	dexamethasone
DN	double negative thymocytes ($CD4^-CD8^-$)
DP	double positive thymocytes ($CD4^+CD8^+$)
DR	diet-restricted group
ER	energy-restricted group
FALS	forward light scatter
FITC	fluorescein isothiocyanate
IFN	interferon
IL	interleukin
MHC	major histocompatibility complex
MZD	marginally zinc-deficient group
PC5	phycoerythrin-cyanine 5
PE	phycoerythrin
PE-CY7	phycoerythrin-cyanine 7
PerCP	peridininchlorophyll-a protein
SS	side scatter
TCR	T-cell receptor

TNF	tumor necrosis factor
ZD	zinc-deficient group

LIST OF COPYRIGHTED MATERIAL FOR WHICH PERMISSION WAS OBTAINED

Hosea HJ, Rector ES, Taylor CG. Zinc-deficient rats have fewer recent thymic emigrant (CD90+) T-lymphocytes in spleen and blood. J Nutr. 2003 Dec;133(12):4239-42.

Hosea HJ, Rector ES, Taylor CG. Age-related changes in p56lck protein levels and phenotypic distribution of T-lymphocytes in young rats. Clin Dev Immunol. 2005 March;12(1):75-84.

Hosea HJ, Rector ES, Taylor CG. Dietary repletion can replenish reduced T-cell subset numbers and lymphoid organ weight in zinc-deficient and energy-restricted rats. B J Nutr. 2004 May;91(5):741-7.

1. GENERAL INTRODUCTION

1.1 Introduction

Nutrition plays an important role in the functioning of the immune system (1). All cells in the body require certain nutrients to function properly, and the highly proliferative cells involved in immune defense are particularly vulnerable to nutrient deficiencies which could leave them functionally compromised (2).

This thesis explores the essentiality of zinc in the development and proper functioning of the immune system, focusing particularly on the T-cells which are involved in the cell-mediated immune response. Studies have shown that dietary zinc deficiency results in a decreased ability to fight off infection due to a decrease in T-cell numbers and a decreased function of the existing T-cells (3). It has been hypothesized that elevated stress hormone levels in dietary zinc deficiency explain the lymphopenia (4). Other research has also pointed to a potential signaling defect (elevated $p56^{lck}$ protein levels) in dietary zinc deficiency that might explain both the lymphopenia and reduced function of the T-cells (5). However, the mechanism that explains the immunodeficiency of dietary zinc deficiency has not been established conclusively.

This thesis will examine the interrelationships among the hypothalamus-pituitary-adrenal stress axis, $p56^{lck}$ protein levels, dietary zinc deficiency and repletion, and their effects on T-lymphocyte maturation and apoptosis (programmed cell death). The overall implication of this work is a deeper understanding of the role of dietary zinc in the highly complicated development and functioning of T-cells in the growing animal. This work contributes a new

hypothesis to explain the immunodeficiency associated with dietary zinc deficiency: fewer recent thymic emigrants over time will limit the T-cell repertoire, leaving the subject more susceptible to infection. The relevant literature is discussed in the following chapter.

1.2 Literature Cited

1. Cunningham-Rundles S, McNeeley DF, Moon A. Mechanisms of nutrient modulation of the immune response. J Allergy Clin Immunol. 2005 Jun;115(6):1119,28; quiz 1129.

2. Field CJ, Johnson IR, Schley PD. Nutrients and their role in host resistance to infection. J Leukoc Biol. 2002 Jan;71(1):16-32.

3. Shi HN, Scott ME, Stevenson MM, Koski KG. Energy restriction and zinc deficiency impair the functions of murine T cells and antigen-presenting cells during gastrointestinal nematode infection. J Nutr. 1998 Jan;128(1):20-7.

4. Fraker PJ. Roles for cell death in zinc deficiency. J Nutr. 2005 Mar;135(3):359-62.

5. Taylor CG, Giesbrecht JA. Dietary zinc deficiency and expression of T lymphocyte signal transduction proteins. Can J Physiol Pharmacol. 2000 Oct;78(10):823-8.

2. LITERATURE REVIEW

2.1 Zinc

2.1.1 Functional roles of zinc

Zinc is known to play a role structurally or functionally in over 300 enzymes in the body and is also involved in two other classes of proteins: metallothioneins and gene regulatory proteins (1). In the cell, zinc containing proteins play a role in everything from metabolic pathways to regulation of gene expression (2). One important group of zinc proteins are 'zinc fingers'. Zinc finger proteins are aptly named because zinc stabilizes small protein loops that resemble fingers, which allows the zinc finger to bind to DNA or other proteins (3). Zinc finger proteins are found throughout the cell and may play a part in countless protein to protein interactions including signal transduction in T-cells (4). Since zinc is part of so many proteins necessary for signal transduction and proliferation, it comes as no surprise that dietary zinc has an influence on the highly proliferative immune system (5).

2.1.2 Zinc body pools

Plasma contains approximately 1 μg zinc/ml bound to α-macroglobulin and albumin, and this represents <1% of total body zinc (1). During zinc deficiency in weanling rats, plasma zinc concentration drops from around 16 to 6 μmol/L (6). However, plasma zinc concentrations can not be reliably used to diagnose zinc deficiency because it is not sensitive enough to detect marginal zinc deficiency and it is not specific, because acute infections can also lower plasma zinc concentrations (7). The other 99% of total body zinc is intracellular

(1). Intracellular zinc is often bound to metallothionein which has a short half-life, leading some to believe that it acts as a zinc pool (6). However, there is no concrete evidence that any one tissue stores zinc. Some have looked to bone as a potential zinc pool, since bone holds 30% of total body zinc (8). Evidence shows that during initial zinc deficiency, there is a small pool of zinc that can be removed from bone (10-20%), and after that no more zinc can be removed without losing bone mass (8). Femur zinc concentration is often used to evaluate long term zinc deficiency in animal models.

2.1.3 Dietary zinc deficiency

Dietary zinc deficiency occurs when there is an inadequate dietary zinc intake or a decreased ability to absorb zinc from the diet (9). Because zinc is present in a wide variety of foods (particularly protein rich foods) dietary zinc deficiency was not a concern until the first documented cases of dietary zinc deficiency were reported in the 1960s (10). A group of teenaged Middle Eastern boys presented with dwarfism, delayed sexual maturation, rough dry skin and increased susceptibility to infections (11). These boys were not related, and the only commonality between them was a cereal based diet that was both low in zinc and high in phytates. Phytates, found in whole grain cereals, pulses and nuts, inhibit the absorption of zinc thereby increasing the risk of dietary zinc deficiency (12).

Although dietary zinc deficiency was originally thought to be a rare occurrence, it has recently been estimated that 1 out of 5 people in the world might not be consuming or absorbing enough zinc from their diet (13). Studies

from around the world have shown that zinc supplementation reduces the rates of diarrhea, pneumonia, malaria and mortality in children (14-17) suggesting that a small increase in dietary zinc intake could make a difference in the health of millions of people in this world. Besides dietary intake, there are diseases that affect the absorption of zinc from the diet which contribute to the prevalence of zinc deficiency including renal disease, Crohn's disease, sickle cell anemia and acrodermatitis enteropathica (18).

Zinc deficiency is listed as one of the top 10 risk factors of disease in developing countries and among the top 20 on a global scale (19). Zinc deficiency is a public health concern because it alters immune function leaving the population susceptible to disease. The research designed to elucidate how zinc deficiency results in immunodeficiency will be discussed in upcoming sections. In order to understand the role of zinc in the immune system, a general discussion of the immune system is offered in the following section.

2.2 The Immune System

2.1.1 The immune response

Our bodies provide the perfect environment for cells to live and function, including pathogens. The evolution of the immune system provides us with a complex mechanism to protect us from death by infection (20). Various analogies can be drawn between the components of the immune system and various defenses for protection of one's home or property. The first line of defense against foreign antigens is the skin and mucous membranes that work

much like the walls, windows and doors of a house that keep strangers on the street from entering the home (21). Often homes are armed with an alarm system that sounds whenever an intruder enters. The alarm system is similar to the second line of defense called the innate immune response, which will respond to all foreign antigens nonspecifically (22). For example, a cut in the skin allows microorganisms to invade the body and the immune system initiates an inflammatory response which summons the arrival of immune cells to destroy the pathogen (22). The immune cells that arrive recognize that the pathogen is foreign, but they don't need to know that pathogen specifically (i.e. will respond similarly whether the pathogen is the Staphylococcus or the West Nile Virus). Humans have evolved a third line of defense, called the adaptive immune response, whereby the immune cells recognize one specific pathogen (20). This is like the fire alarm in a house, which will only sound when a fire has started and summons the appropriate aid.

The adaptive immune response is mediated by B- and T-lymphocytes (or B- and T-cells) (21). B-cells mature in the bone marrow, while T-cells leave the bone marrow to mature in the thymus before entering secondary lymphoid organs like the spleen, lymph nodes and gut associated lymphoid tissue where they respond to their specific antigen when stimulated (23).

Activation of B-cells results in the production of antibodies which then travel throughout the body and bind to all molecules that are similar to the antigen that initiated the immune response (23). Once the antibodies have bound the antigens, this either inactivates the antigen, binds antigens together to

facilitate phagocytosis by macrophages or destroys the antigen by activating the complement cascade (20). This response is called the humoral response (20).

T-cells attack antigens more directly and make up what is called the cell-mediated response (20). T-cells have two main subsets: cytotoxic and helper T-cells (24). Determination of cell surface markers or clusters of differentiation (CD) using monoclonal antibodies and flow cytometry can distinguish the subtype of lymphocyte. CD4 can be used to identify helper T-cells while CD8 reveals cytotoxic T-cells (23).

Some of the classic characteristics of dietary zinc deficiency are thymic atrophy and lymphopenia which has focused attention on T-cells (5) and the rest of the literature review will pertain directly to this subset of immune cells.

2.2.2 T-cell maturation

The thymus gland is a bi-lobed organ situated above the heart. Each lobe can be divided into lobules and each lobule can be divided into an outer area or cortex and an inner area or medulla (25). The most immature T-cells are found in the cortex and do not express the T-cell receptor (TCR):CD3 complex, CD4 or CD8 (26). As the cells travel towards the medulla, they begin to express TCR$\alpha\beta$, CD4, and CD8 (26). Before thymocytes become fully mature and express either CD4 or CD8 along with TCR$\alpha\beta$, they go through positive and negative selection (27). This selection process ensures that cells that react strongly to self major histocompatibility complexes (MHC) are removed by apoptosis and those that don't recognize self MHC at all die of neglect (24). The surviving thymocytes leave the thymus and seed the periphery with "naïve" T-cells capable of further

self-replication. Following appropriate antigenic stimulation, memory T-cells are formed (24). Homeostasis of the T-cell compartment is maintained when there is control over the number of cells being made and the number of cells being destroyed (28). If too many cells are killed, leukopenia and immunodeficiency occurs (28). If not enough lymphocytes are destroyed, autoimmunity or cancer can occur (28). A malfunctioning thymus can prevent production or affect the function of mature T-cells.

Intrathymic labeling using fluorescein isothiocyanate (FITC) (29) and thymus transplantation (30) have allowed researchers to identify and characterize differentiation of T-lymphocytes once they have been released from the thymus into the periphery. CD90 first appears on cortical thymocytes and is found on newly released T-cells (recent thymic emigrants) for approximately 3 days in rats (29). The ability to identify newly released thymocytes with CD90 is unique to the rat, and therefore, positions the rat as the best model to study early post-thymic development. CD90 plays a role in T-cell activation (31), and in the diabetes-prone BioBreeding rat a high proportion of recent thymic emigrants are apoptotic (32). RT6 and CD45RC are expressed as CD90 disappears (3-11 days post-thymus) and 76 days after T-cells have been released from the thymus they express either CD45RC or RT6 (30). CD45RC is involved in T- and B-cell receptor signal transduction, and is often used to discriminate naïve from memory T-cells (33, 34). RT6.1 is expressed solely on peripheral T-cells and it is hypothesized that RT6.1 expression prevents apoptosis of recent thymic emigrants (35). Further characterization of T-cells using CD90, CD45RC, and

RT6.1 would be of interest in determining how dietary zinc deficiency alters the T-cell population.

2.2.3 Characterization of T-cells by flow cytometry

The rapid measurement of the physical and/or fluorescence characteristics of individual cells while they pass, in single file, through a laser beam of light is called flow cytometry (36). As the cells pass through the laser, they scatter light in two ways. Forward scatter is the light deflected from the surface of the cell and indicates the size of the cell, while side scatter is either the light deflected off internal structures of the cell, or light from the florescent dye (37). Cells are most often labeled using monoclonal antibodies that are directly bound to fluorochromes, or indirectly when a fluorochrome is conjugated to a secondary antibody that recognizes an unconjugated antibody (36). There are numerous flurochromes available, each producing a distinct color that can be used in combination to determine many characteristics of the cell simultaneously (38). Multiparametric flow cytometry is often used to identify the phenotype of cells based on their cell surface markers (i.e. CD4 vs. CD8) and report the proportion of these cells in a sample population. In addition, some flow cytometers can also isolate or sort cells of interest into purified populations for further study (38). Intracellular staining of DNA to generate cell cycle histograms has been used to identify apoptotic cells with fragmented DNA which appear in the hypodiploid region of the cell cycle hisotogram (39). In combination with cell surface staining, intracellular staining of DNA allows investigators to determine the proportion of apoptotic cells among cell populations (39).

2.2.4 T-cell function

Functional assays of T-cell function include measuring proliferation, cytokine production, and cytotoxicity (40). A characteristic of the acquired immune response is the ability of lymphocytes specific to an antigen to proliferate and differentiate upon activation, which is often referred to as clonal selection (20). Upon activation, helper T-cells also produce and secrete cytokines that stimulate the activity of other immune cells, while cytotoxic T-cells kill the target cell when activated (23). For activation, cytotoxic T-cells require the antigen to be combined with class I MHC, which are present on all nucleated cells (24). An antigen activates the TCR on helper T-cells only when it is presented as a complex with a MHC class II protein, which is expressed by antigen presenting cells like B-cells, macrophages and dendritic cells (24).

Helper T-cells can also be classified based on the cytokines they produce. Th1 cells produce interleukin-2 (IL-2) and interferon-γ (IFN–γ), which are effective at stimulating the cell mediated immune response to fight off intracellular invaders (e.g. viruses)(41). Th2 cells produce IL-4 and IL-10 which stimulate the humoral immune response which is more effective for fighting off intercellular pathogens (e.g. worms) (41). The cytokines produced by Th1 and Th2 cells inhibit each other and ensure that the immune response is not excessive (42). Therefore, an imbalance in the Th1 to Th2 responses could result in autoimmune diseases from an uncontrolled immune response (43).

2.3 Dietary Zinc Deficiency and Cell-Mediated Immunity

2.3.1 Pair-fed controls

Studies on the impact of dietary zinc deficiency on the immune system have been done mostly using the adult mouse as a model (44). A potential confounding factor in studies examining the effects of dietary zinc deficiency on the immune system is that the consumption of a zinc deficient diet results in significant reductions in feed intake which, in turn, produces stunting malnutrition in growing animals (45) or wasting malnutrition in adult rodents (46). Energy malnutrition has a negative impact on the ability of the body to fight off disease (47); therefore, an additional control must be included in research studies to separate out the effects of malnutrition from zinc deficiency. The "pair-fed" animal is individually matched to a zinc deficient animal. The pair-fed animal is provided the control diet, but limited to the amount of feed as consumed by its zinc deficient paired mate on the previous day to control for the effects of malnutrition (48).

2.3.2 Zinc deficiency and lymphopenia

Like humans, zinc deficient mice are less able to fight off infection compared to zinc adequate controls (49, 50). Fraker and colleagues found that zinc deficient mice weighed 30% less than controls, and that their spleen and thymus weighed 50 and 70% less than controls, respectively (51). Reduced lymphoid organ weight suggests reduced cell numbers which theoretically would result in fewer immune cells available to fight off infection. More recent studies

have shown that zinc-deficient mice do indeed have fewer splenocytes (50) and thymocytes (52) which could contribute to the immunodeficiency.

2.3.3 Zinc deficiency and T-cell function

It was also of interest to determine whether the reduced ability to fight off infection was due solely to lower lymphocyte numbers, or if the functional ability of the existing cells was also compromised in zinc deficiency. A study by Shi and colleagues (50) has shown on a per cell basis lower proliferation rates and cytokine production by T-cells in response to antigen in zinc deficiency compared to a pair-fed group and *ad libitum* fed controls. This provides evidence that not only are lymphocyte numbers reduced, but their ability to respond to antigens is also compromised in dietary zinc deficiency.

2.3.4 Zinc deficiency and T-cell phenotypes

Although the function of T-cells was reduced, the question remained whether zinc deficient mice had an altered proportion of immune cell phenotypes which might explain the reduced functional abilities of the cells, or if the reduction in lymphocyte numbers was balanced across all cell types. Using CD3, CD4, and CD8 to identify T-cell subsets, no differences in the proportions of T-cell phenotypes were found among dietary treatment groups for thymocytes (53) or splenocytes (50) from zinc deficient mice. In experimental zinc restriction in humans, there was a decrease in the ratio of CD4:CD8 and a trend ($p=0.077$, $n=5$) for a lower ratio of $CD4^+CD45RA^+$ (naïve) to $CD4^+CD45RO^+$ (memory) cells in the blood of the volunteers (54). Post-thymic changes in T-cell phenotypes using markers like CD90, CD45 (RA in humans, RB in mice, RC in rats) and

RT6.1 have not been examined in dietary zinc deficiency, but would be of great interest to elucidate the mechanism behind the immune impairment in dietary zinc deficiency.

2.3.5 Zinc deficiency and corticosterone

A theoretical explanation for the lymphopenia that characterizes zinc deficiency discussed in the literature involves corticosterone produced during stimulation of the hypothalamus-pituitary-adrenal stress axis (55). High levels of corticosterone from the adrenals can cause involution of the thymus by inducing apoptosis in thymocytes (56). Apoptosis or programmed cell death is a form of cell suicide. Apoptosis occurs in an organized fashion whereby the nucleus and cytoplasm condense, chromatin is cleaved, and apoptotic bodies are removed by lysosomes without causing any significant inflammation (28). Necrosis is another form of cell death that occurs when cells take up water until the plasma membrane bursts releasing the contents of the cell resulting in the initiation of an immune response (28). Depasquale-Jardieu and Fraker (57) hypothesized that dietary zinc deficiency creates a stress in the animal by increasing the level of serum corticosteroids, and consequently resulting in lower numbers of functional thymocytes due to apoptosis. They reported that the zinc deficient mice had enlarged adrenal glands and higher plasma corticosterone concentrations compared to *ad libitum* fed controls (3.3 μM vs 1.2 μM, respectively) (57). A recent report showed that zinc deficient mice had an increased proportion of apoptotic thymocytes compared to controls (52). However, a limitation of this study is the lack of a pair-fed group, because malnourished mice also have

elevated corticosterone levels compared to controls (58). Therefore, it is not clear if the increased apoptosis is due to high corticosterone concentrations alone or if zinc deficiency has an additional effect. DePasquale-Jardieu and Fraker detected a loss in T-cell helper function in the zinc-deficient mice before any elevation in serum corticosterone suggesting that there are other factors besides the higher corticosterone concentrations associated with the depletion in zinc that are compromising immune function (57). Removal of adrenal glands did not offer significant protection against the immune dysfunction observed in zinc-deficient mice providing further evidence that corticosterone is not the only cause of the immunodeficiency in zinc-deficient mice (59).

2.3.6 Zinc deficiency and $p56^{lck}$

Another possible mechanism that could explain both the decreased number and decreased function of T-cells in zinc deficiency is an increased expression of the zinc finger signaling protein $p56^{lck}$ (4). $P56^{lck}$ is only expressed in lymphoid cells, and almost solely in T-cells (60). $P56^{lck}$ is part of the src-protein family, which is associated with the inside of the plasma membrane and CD4 or CD8 (61). The cytoplasmic domains of CD4 and CD8 interact with the N-terminal of $p56^{lck}$ through their cysteine residues (62). The pairs of cysteine residues form a zinc-binding site, suggesting that zinc binding is necessary to stimulate $p56^{lck}$ phosphorylation (63). It is believed that once CD4 or CD8 stimulates $p56^{lck}$, it phosphorylates the CD3 augmenting the TCR signals (64). If the phosphorylation of this protein is inhibited, there is no activation of the T-cell (65).

Genetically altered mice missing $p56^{lck}$ (lcknull) or those producing a catalytically inactive form of $p56^{lck}$ (lckR257 transgenic mice) have thymic atrophy and a decreased number of thymocytes (66, 67). In both kinds of genetically altered mice there was a block in the maturation of thymocytes from double negative ($CD4^-CD8^-$; the most immature) to double positive thymocytes ($CD4^+CD8^+$) suggesting that $p56^{lck}$ plays a role in the maturation of thymocytes (66, 67). There was also a decrease in the number of peripheral T-cells, but the peripheral lymphoid tissues were not as severely affected as the thymus. Molina and colleagues (66) noted that B-cell function was not compromised in the lcknull mice. Levin and colleagues (67) found that thymocytes in lckR273 mice were arrested at a particular point in maturation and were unable to replicate past this point, suggesting that the decreased number of thymocytes was due to less production and not more apoptosis.

$P56^{lck}$ has also been shown to be involved in the signaling of apoptosis. Di Somma and colleagues (68) found that a Jurkat cell line defective for $p56^{lck}$ was resistant to apoptosis, while another cell line with an active form of $p56^{lck}$ that could not be turned off (constitutively expressed) enhanced the sensitivity of Jurkat cells to apoptosis induced by TCR crosslinking.

Researchers have found increased $p56^{lck}$ levels in thymic and splenic T-cells from zinc deficient animals (53, 69). However, the activity of $p56^{lck}$ was not determined. The increased $p56^{lck}$ levels could possibly be an attempt to compensate for decreased function of this protein, and decreased activity of $p56^{lck}$ would explain the compromised functioning of T-cells in dietary zinc

deficiency. However, if the elevated $p56^{lck}$ levels do result in more activity of the protein, then this would leave the T-cells more susceptible to apoptosis. To date, no one has established an association between $p56^{lck}$ and apoptosis in dietary zinc deficiency.

2.4 Literature Cited

1. Vallee BL, Falchuk KH. The biochemical basis of zinc physiology. Physiol Rev. 1993 Jan;73(1):79-118.

2. Coleman JE. Zinc proteins: Enzymes, storage proteins, transcription factors, and replication proteins. Annu Rev Biochem. 1992;61:897-946.

3. Klug A, Schwabe JW. Protein motifs 5. zinc fingers. FASEB J. 1995 May;9(8):597-604.

4. Taylor CG, Giesbrecht JA. Dietary zinc deficiency and expression of T lymphocyte signal transduction proteins. Can J Physiol Pharmacol. 2000 Oct;78(10):823-8.

5. Fraker PJ, King LE, Laakko T, Vollmer TL. The dynamic link between the integrity of the immune system and zinc status. J Nutr. 2000 May;130(5S Suppl):1399S-406S.

6. Bettger WJ, O'Dell BL. Physiological roles of zinc in the plasma membrane of mammalian cells. J Nutr Biochem. 1993;4:194-203.

7. Hambidge M. Biomarkers of trace mineral intake and status. J Nutr. 2003 Mar;133 Suppl 3:948S-55S.

8. Zhou JR, Canar MM, Erdman JW,Jr. Bone zinc is poorly released in young, growing rats fed marginally zinc-restricted diet. J Nutr. 1993 Aug;123(8):1383-8.

9. Prasad AS. Zinc deficiency in women, infants and children. J Am Coll Nutr. 1996 Apr;15(2):113-20.

10. Hambidge M. Human zinc deficiency. J Nutr. 2000 May;130(5S Suppl):1344S-9S.

11. Prasad AS, Miale A,Jr, Farid Z, Sandstead HH, Schulert AR. Zinc metabolism in patients with the syndrome of iron deficiency anemia, hepatosplenomegaly, dwarfism, and hypognadism. J Lab Clin Med. 1963 Apr;61:537-49.

12. Sandstrom B. Bioavailability of zinc. Eur J Clin Nutr. 1997 Jan;51 Suppl 1:S17-9.

13. Wuehler SE, Peerson JM, Brown KH. Use of national food balance data to estimate the adequacy of zinc in national food supplies: Methodology and regional estimates. Public Health Nutr. 2005 Oct;8(7):812-9.

14. Umeta M, West CE, Haidar J, Deurenberg P, Hautvast JG. Zinc supplementation and stunted infants in ethiopia: A randomised controlled trial. Lancet. 2000 Jun 10;355(9220):2021-6.

15. Ninh NX, Thissen JP, Collette L, Gerard G, Khoi HH, Ketelslegers JM. Zinc supplementation increases growth and circulating insulin-like growth factor I (IGF-I) in growth-retarded vietnamese children. Am J Clin Nutr. 1996 Apr;63(4):514-9.

16. Shankar AH, Genton B, Baisor M, Paino J, Tamja S, Adiguma T, Wu L, Rare L, Bannon D, et al. The influence of zinc supplementation on morbidity due to plasmodium falciparum: A randomized trial in preschool children in papua new guinea. Am J Trop Med Hyg. 2000 Jun;62(6):663-9.

17. Sazawal S, Black RE, Jalla S, Mazumdar S, Sinha A, Bhan MK. Zinc supplementation reduces the incidence of acute lower respiratory infections in infants and preschool children: A double-blind, controlled trial. Pediatrics. 1998 Jul;102(1 Pt 1):1-5.

18. Maret W, Sandstead HH. Zinc requirements and the risks and benefits of zinc supplementation. J Trace Elem Med Biol. 2006;20(1):3-18.

19. Guilbert JJ. The world health report 2002 - reducing risks, promoting healthy life. Educ Health (Abingdon). 2003 Jul;16(2):230.

20. Thibodeau GA, Patton KT. Anatomy & physiology. 5th ed. St. Louis, Mo.: Mosby; 2002.

21. Hulsewe KW, van Acker BA, von Meyenfeldt MF, Soeters PB. Nutritional depletion and dietary manipulation: Effects on the immune response. World J Surg. 1999 Jun;23(6):536-44.

22. Erickson KL, Medina EA, Hubbard NE. Micronutrients and innate immunity. J Infect Dis. 2000 Sep;182 Suppl 1:S5-10.

23. Delves PJ, Roitt IM. The immune system. first of two parts. N Engl J Med. 2000 Jul 6;343(1):37-49.

24. Schepers K, Arens R, Schumacher TN. Dissection of cytotoxic and helper T cell responses. Cell Mol Life Sci. 2005 Dec;62(23):2695-710.

25. Savino W. The thymus is a common target organ in infectious diseases. PLoS Pathog. 2006 Jun;2(6):e62.

26. Savino W, Dardenne M. Neuroendocrine control of thymus physiology. Endocr Rev. 2000 Aug;21(4):412-43.

27. Schwartz RS. Shattuck lecture: Diversity of the immune repertoire and immunoregulation. N Engl J Med. 2003 Mar 13;348(11):1017-26.

28. Rathmell JC, Thompson CB. The central effectors of cell death in the immune system. Annu Rev Immunol. 1999;17:781-828.

29. Hosseinzadeh H, Goldschneider I. Recent thymic emigrants in the rat express a unique antigenic phenotype and undergo post-thymic maturation in peripheral lymphoid tissues. J Immunol. 1993 Mar 1;150(5):1670-9.

30. Kampinga J, Groen H, Klatter FA, Pater JM, van Petersen AS, Roser B, Nieuwenhuis P, Aspinall R. Post-thymic T-cell development in the rat. Thymus. 1997;24(3):173-200.

31. Loertscher R, Lavery P. The role of glycosyl phosphatidyl inositol (GPI)-anchored cell surface proteins in T-cell activation. Transpl Immunol. 2002 May;9(2-4):93-6.

32. Iwakoshi NN, Goldschneider I, Tausche F, Mordes JP, Rossini AA, Greiner DL. High frequency apoptosis of recent thymic emigrants in the liver of lymphopenic diabetes-prone BioBreeding rats. J Immunol. 1998 Jun 15;160(12):5838-50.

33. Xystrakis E, Bernard I, Dejean AS, Alsaati T, Druet P, Saoudi A. Alloreactive CD4 T lymphocytes responsible for acute and chronic graft-versus-host disease are contained within the CD45RChigh but not the CD45RClow subset. Eur J Immunol. 2004 Feb;34(2):408-17.

34. Bunce C, Bell EB. CD45RC isoforms define two types of CD4 memory T cells, one of which depends on persisting antigen. J Exp Med. 1997 Feb 17;185(4):767-76.

35. Thiele HG, Haag F. The RT6 system of the rat: Developmental, molecular and functional aspects. Immunol Rev. 2001 Dec;184:96-108.

36. Delude RL. Flow cytometry. Crit Care Med. 2005 Dec;33(12 Suppl):S426-8.

37. Darzynkiewicz Z, Bedner E, Smolewski P. Flow cytometry in analysis of cell cycle and apoptosis. Semin Hematol. 2001 Apr;38(2):179-93.

38. De Rosa SC, Brenchley JM, Roederer M. Beyond six colors: A new era in flow cytometry. Nat Med. 2003 Jan;9(1):112-7.

39. Fraker PJ, King LE, Lill-Elghanian D, Telford WG. Quantification of apoptotic events in pure and heterogeneous populations of cells using the flow cytometer. Methods Cell Biol. 1995;46:57-76.

40. Field CJ. Using immunological techniques to determine the effect of nutrition on T-cell function. Can J Physiol Pharmacol. 1996 Jun;74(6):769-77.

41. DiPiro JT. Cytokine networks with infection: Mycobacterial infections, leishmaniasis, human immunodeficiency virus infection, and sepsis. Pharmacotherapy. 1997 Mar-Apr;17(2):205-23.

42. Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. Immunol Today. 1996 Mar;17(3):138-46.

43. Sprietsma JE. Zinc-controlled Th1/Th2 switch significantly determines development of diseases. Med Hypotheses. 1997 Jul;49(1):1-14.

44. Fraker PJ, King LE. Reprogramming of the immune system during zinc deficiency. Annu Rev Nutr. 2004;24:277-98.

45. Giugliano R, Millward DJ. Growth and zinc homeostasis in the severely zn-deficient rat. Br J Nutr. 1984 Nov;52(3):545-60.

46. Cook-Mills JM, Fraker PJ. Functional capacity of the residual lymphocytes from zinc-deficient adult mice. Br J Nutr. 1993 May;69(3):835-48.

47. Woodward B. Protein, calories, and immune defenses. Nutr Rev. 1998 Jan;56(1 Pt 2):S84-92.

48. Panemangalore M, Bebe FN. Zinc diets: Deficiency and excess. In: Watson RR, editor. Trace elements in laboratory rodents. Boca Ratton, Florida: CRC press; 1997. p. 191-214.

49. Fraker PJ, Caruso R, Kierszenbaum F. Alteration of the immune and nutritional status of mice by synergy between zinc deficiency and infection with trypanosoma cruzi. J Nutr. 1982 Jun;112(6):1224-9.

50. Shi HN, Scott ME, Stevenson MM, Koski KG. Energy restriction and zinc deficiency impair the functions of murine T cells and antigen-presenting cells during gastrointestinal nematode infection. J Nutr. 1998 Jan;128(1):20-7.

51. Fraker PJ, DePasquale-Jardieu P, Zwickl CM, Luecke RW. Regeneration of T-cell helper function in zinc-deficient adult mice. Proc Natl Acad Sci U S A. 1978 Nov;75(11):5660-4.

52. King LE, Osati-Ashtiani F, Fraker PJ. Apoptosis plays a distinct role in the loss of precursor lymphocytes during zinc deficiency in mice. J Nutr. 2002 May;132(5):974-9.

53. Moore JB, Blanchard RK, McCormack WT, Cousins RJ. cDNA array analysis identifies thymic LCK as upregulated in moderate murine zinc deficiency before T-lymphocyte population changes. J Nutr. 2001 Dec;131(12):3189-96.

54. Beck FW, Prasad AS, Kaplan J, Fitzgerald JT, Brewer GJ. Changes in cytokine production and T cell subpopulations in experimentally induced zinc-deficient humans. Am J Physiol. 1997 Jun;272(6 Pt 1):E1002-7.

55. Fraker PJ, Osati-Ashtiani F, Wagner MA, King LE. Possible roles for glucocorticoids and apoptosis in the suppression of lymphopoiesis during zinc deficiency: A review. J Am Coll Nutr. 1995 Feb;14(1):11-7.

56. Hadden JW. Thymic endocrinology. Ann N Y Acad Sci. 1998 May 1;840:352-8.

57. DePasquale-Jardieu P, Fraker PJ. The role of corticosterone in the loss in immune function in the zinc-deficient A/J mouse. J Nutr. 1979 Nov;109(11):1847-55.

58. Monk JM, Makinen K, Shrum B, Woodward B. Blood corticosterone concentration reaches critical illness levels early during acute malnutrition in the weanling mouse. Exp Biol Med (Maywood). 2006 Mar;231(3):264-8.

59. DePasquale-Jardieu P, Fraker PJ. Further characterization of the role of corticosterone in the loss of humoral immunity in zinc-deficient A/J mice as determined by adrenalectomy. J Immunol. 1980 Jun;124(6):2650-5.

60. Perlmutter RM, Marth JD, Ziegler SF, Garvin AM, Pawar S, Cooke MP, Abraham KM. Specialized protein tyrosine kinase proto-oncogenes in hematopoietic cells. Biochim Biophys Acta. 1989 Feb;948(3):245-62.

61. Veillette A, Bookman MA, Horak EM, Bolen JB. The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase p56lck. Cell. 1988 Oct 21;55(2):301-8.

62. Turner JM, Brodsky MH, Irving BA, Levin SD, Perlmutter RM, Littman DR. Interaction of the unique N-terminal region of tyrosine kinase p56lck with cytoplasmic domains of CD4 and CD8 is mediated by cysteine motifs. Cell. 1990 Mar 9;60(5):755-65.

63. Pernelle JJ, Creuzet C, Loeb J, Gacon G. Phosphorylation of the lymphoid cell kinase p56lck is stimulated by micromolar concentrations of Zn2+. FEBS Lett. 1991 Apr 9;281(1-2):278-82.

64. Weil R, Veillette A. Signal transduction by the lymphocyte-specific tyrosine protein kinase p56lck. Curr Top Microbiol Immunol. 1996;205:63-87.

65. June CH, Fletcher MC, Ledbetter JA, Schieven GL, Siegel JN, Phillips AF, Samelson LE. Inhibition of tyrosine phosphorylation prevents T-cell receptor-mediated signal transduction. Proc Natl Acad Sci U S A. 1990 Oct;87(19):7722-6.

66. Molina TJ, Kishihara K, Siderovski DP, van Ewijk W, Narendran A, Timms E, Wakeham A, Paige CJ, Hartmann KU, Veillette A. Profound block in thymocyte development in mice lacking p56lck. Nature. 1992 May 14;357(6374):161-4.

67. Levin SD, Anderson SJ, Forbush KA, Perlmutter RM. A dominant-negative transgene defines a role for p56lck in thymopoiesis. EMBO J. 1993 Apr;12(4):1671-80.

68. Di Somma MM, Nuti S, Telford JL, Baldari CT. p56lck plays a key role in transducing apoptotic signals in T cells. FEBS Lett. 1995 Apr 17;363(1-2):101-4.

69. Lepage LM, Giesbrecht JA, Taylor CG. Expression of T lymphocyte p56(lck), a zinc-finger signal transduction protein, is elevated by dietary zinc deficiency and diet restriction in mice. J Nutr. 1999 Mar;129(3):620-7.

3. RATIONALE

3.1 Exploration of Potential Mechanisms behind Immunodeficiency

Studies using the adult mouse as a model have shown that dietary zinc deficiency results in a decreased ability to fight infection in part due to decreased lymphocyte numbers and in part due to a decreased functional ability of the residual cells (1). At the molecular level, the zinc finger signaling protein $p56^{lck}$ is elevated in zinc deficiency (2, 3) but the functional outcome of this (i.e. defective thymocyte maturation or increased apoptotic susceptibility of T-cells) has not been confirmed. Increased thymocyte apoptosis rates in zinc deficient adult mice have been linked to elevated corticosterone concentrations (4); however, the role of the hypothalamus-pituitary-adrenal stress axis in immunodeficiency has not been explored in the growing rat model of zinc deficiency.

Cytokines play a role in thymocyte development (5) and peripheral T-cell activities (6). Cytokine production by splenocytes has been shown to be altered in dietary zinc deficient humans (7), mice (1) and cell lines (8), but it has not been determined in the growing rat and the effect of zinc deficiency on thymocyte cytokine production has not been examined in any model of zinc deficiency. Further characterization of T-cell maturation beyond the usual TCR$\alpha\beta$/CD3, CD4, CD8, and CD45RA (marker of naïve T-cells in humans) expression has not been done in dietary zinc-deficiency. CD90, RT6.1 and CD45RC can be used to further characterize the stage of maturity of the peripheral T-cells in the zinc deficient rat. If dietary zinc deficiency affects the proportions of T-cells at various stages of post-thymic development, then it would be of interest to determine

whether the differences are due to increased removal of certain subsets via apoptosis. Proportions along with absolute cell numbers would provide a more complete picture of the status of the T-cell subsets in zinc deficiency.

3.2 Animal Model

The World Health Report in 2002 identified zinc deficiency as one of the major causes of disease, especially in developing countries (9). Children are one of the segments of the population that are at particular risk (9). However, studies investigating the impact of dietary zinc deficiency on the immune system have largely been done using the adult mouse as a model (10). The mouse has been a convenient model because the necessary antibodies were readily available. However, mice are not amenable to studying the effects of dietary treatments during growth because of they achieve adult size quickly and there is relatively little difference between body weight during infancy and adulthood (11). Another limitation of their size is it can be difficult to remove enough tissue, including blood, from one animal to use for all the desired laboratory tests.

The use of the growing rat model for studies of dietary zinc deficiency on T-cell development/function is now possible due to increased availability of the necessary antibodies. The use of the growing rat as a model in dietary zinc deficiency allows the researcher to explore the effects of this nutritional deficiency at an earlier stage of development. Rats can provide more tissue per animal, which allows for multiple tests on one animal, providing a more holistic picture of what it happening *in vivo*. It is of great interest to identify and examine

recent thymic emigrants since it remains unclear whether the immunodeficiency of dietary zinc deficiency is due to a defect in T-cell maturation in the thymus or in the periphery. The rat offers the unique opportunity to explore the effect of dietary zinc deficiency on the newly produced T-cells using CD90 as a marker, since no such cell surface marker has been identified in mice or humans (12).

3.3 Treatment Groups

The inclusion of a pair-fed or diet-restricted group is critical in studies of dietary zinc deficiency to isolate the effects of zinc deficiency from the accompanying malnutrition (13). Another valuable control is a marginally zinc-deficient group, which receives a diet low in zinc, but not sufficient to induce anorexia and weight loss. The marginally zinc-deficient group in combination with the pair-fed group can be used to separate the effects of low zinc status from malnutrition. Because severe zinc deficiency is rare in the human population, the marginal zinc deficient group represents the degree of zinc deficiency which is more prevalent.

The dietary zinc levels for the control diet (30 mg Zn/kg diet) were based on the American Institute of Nutrition-93G recommendations (14). The zinc deficient diet was based on previous studies showing that providing <1 mg Zn/kg diet to growing rats for 3 weeks resulted in severely reduced zinc status (15). A marginally zinc deficient diet of 10 mg Zn/kg diet was based on previous reports that this level of zinc results in reduced zinc status without affecting body weight (Taylor, personal communication). Given that the zinc requirement for rats is 12

mg Zn/kg diet (16) the reduced feed intake (approximately 50%) of the pair-fed group would still result in sufficient zinc intake.

3.4 Selection of Tissues

Studies of dietary zinc deficiency on T-cell development and function should investigate both thymocytes and T-cells from secondary lymphoid organs (e.g. spleen) to determine whether any changes in T-cell subsets occurs during the maturation process in the thymus or once the cells have been released into the periphery. Immunological studies in rodent models should also investigate whether the effects in lymphoid tissue are reflected in immune cells isolated from blood which will help in the translation to humans studies where this is often the only tissue that can be sampled.

3.5 Questions Arising From Gaps in the Literature

- Are the effects of dietary zinc deficiency in the growing rat similar to the adult mouse (i.e. lymphoid organ atrophy, lymphopenia, no change in T-cell phenotypes, elevated $p56^{lck}$ protein levels and serum corticosterone concentrations)?
- Are elevated thymocyte $p56^{lck}$ protein levels in dietary zinc deficiency associated with impaired thymocyte development in the growing rat?
- Is post-thymic development altered by dietary zinc deficiency?
- Does dietary zinc deficiency alter cytokine production by thymocytes or splenocytes?

- Does dietary zinc deficiency leave thymocytes and splenic T-cells more susceptible to apoptosis in the growing rat? And if so, is one particular subset more resistant or vulnerable than another?
- Is it possible for the immune system to recover from an insult like dietary zinc deficiency early in life, and if so what is the time course for recovery?

3.6 Hypotheses and Objectives

3.6.1 T-cell maturation in zinc-deficient rats (Chapter 4)

Hypothesis

Dietary zinc deficiency increases $p56^{lck}$ expression in thymocytes resulting in a large proportion of immature cells and therefore, fewer new T-cells in the periphery (blood and spleen).

Objectives

To examine $p56^{lck}$ protein levels (by western blotting), the proportion of T-cell subsets using flow cytometric markers of T-cell development (CD4, CD8, TCR$\alpha\beta$, TCR$\gamma\delta$, and CD90) and absolute T-cell numbers (using Flow Count™ fluorospheres) in thymus, spleen and blood of zinc-deficient and diet-restricted growing rats.

3.6.2 Immune system recovery after dietary zinc-deficiency in the growing rat (Chapter 5)

Hypothesis

Dietary zinc deficiency in the growing rat, like the adult mouse, will result in lymphoid organ atrophy and lymphopenia and these will recover to control levels through repletion with control diet.

Objectives

To investigate the time course of recovery of lymphoid organ weight and absolute number of lymphocyte subsets (by flow cytometry) from the thymus, spleen and blood of the growing rat through repletion with a zinc adequate diet.

3.6.3 Dietary zinc deficiency and the hypothalamus-pituitary-adrenal stress axis in the growing rat (Chapter 5)

Hypothesis

The lymphopenia (low thymus and spleen T-cell numbers) of dietary zinc deficiency is associated with stimulation of the hypothalamus-pituitary-adrenal stress axis, resulting in high serum adrenocorticotropic hormone (ACTH), corticosterone, tumor necrosis factor (TNF)–α and haptoglobin concentrations compared to diet-restricted and *ad libitum* fed controls.

Objectives

To characterize the effect of dietary zinc deficiency on components of the hypothalamus-pituitary-adrenal stress axis (serum ACTH, corticosterone, TNF–α, and haptoglobin) and lymphoid organs (thymus and spleen) in the growing rat.

3.6.4 Age-related changes in $p56^{lck}$ protein levels and phenotypic distribution of T-lymphocytes in young rats (Chapter 6)

Hypothesis

$P56^{lck}$ protein levels and T-cell subsets will change with age from 3 to 9 weeks of age in the growing rat.

Objective

To determine $p56^{lck}$ protein levels (by western immunonblotting) and T-cell subsets (by flow cytometery) in the thymus, spleen and blood of 3, 6, and 9 week old rats.

3.6.5 Post thymic development in zinc deficient rats (Chapter 7)

Hypothesis

Zinc deficiency alters post thymic development resulting in a lower proportion of new T-cells in the spleen, due to a higher proportion of new T-cells undergoing apoptosis.

Objective

To determine the proportion of lymphocytes that express different combinations of TCR$\alpha\beta$, CD90, RT6.1 and CD45RC in both marginal and severe zinc deficiency using the growing rat as a model.

To determine the proportion of T-cell subsets that are apoptotic or viable using flow cytometry.

3.6.6 Susceptibility to apoptosis of recent thymic emigrants in zinc deficient rats (Chapter 8)

Hypothesis

The reduced proportion of recent thymic emigrant T-cells in zinc-deficient rats is due to increased susceptibility of $CD90^+$ T-cells to apoptosis upon exiting the thymus.

Objective

To determine the proportion of apoptotic cells in the T-cell sub-populations among zinc-deficient, diet-restricted, marginally zinc-deficient and control growing rats.

3.6.7 Cytokine production in zinc-deficient growing rats (Chapter 9)

Hypothesis

Low zinc status during dietary zinc deficiency shifts cytokine production in favor of a Th2 response (less IL-2 and IFN-γ) and this shift in cytokine production occurs in conjunction with higher serum corticosterone concentrations associated with dietary zinc deficiency.

Objective

To determine potential differences in the *in vitro* cytokine production (IL-2, IFN-γ, IL-6, and IL-10) of isolated thymocytes and splenocytes after mitogenic stimulation with concavalin A in zinc-deficient, diet-restricted, marginally zinc-deficient and control rats.

To determine thymic and splenic T-cell subset absolute cell numbers using flow cytometry and Flow-Count™ Fluorospheres, and serum corticosterone concentrations.

Note: The thesis results are presented in manuscripts (Chapters 4-9). I (Heather Hosea) performed all the laboratory work and statistical analysis described therein. I received assistance with animal care from the staff of the Animal Holding Facility, Dr. Carla Taylor, Amy Noto, Laura Burr, Jody Van Deynze and Lisa Rigaux. Dr. Carla Taylor assisted me with collection of tissues for the experiments described in Chapters 4-6, and I collected the tissues described in Chapters 7-9. Jennifer Jamieson assisted in the collection of cell culture supernatants. Dr. Edward Rector operated the flow cytometer and acquired all of the flow cytometry data. I analyzed all of the data derived from the various experimental procedures, wrote the manuscripts and received feedback from the co-authors.

3.7 Literature Cited

1. Shi HN, Scott ME, Stevenson MM, Koski KG. Energy restriction and zinc deficiency impair the functions of murine T cells and antigen-presenting cells during gastrointestinal nematode infection. J Nutr. 1998 Jan;128(1):20-7.

2. Lepage LM, Giesbrecht JA, Taylor CG. Expression of T lymphocyte p56(lck), a zinc-finger signal transduction protein, is elevated by dietary zinc deficiency and diet restriction in mice. J Nutr. 1999 Mar;129(3):620-7.

3. Moore JB, Blanchard RK, McCormack WT, Cousins RJ. cDNA array analysis identifies thymic LCK as upregulated in moderate murine zinc deficiency before T-lymphocyte population changes. J Nutr. 2001 Dec;131(12):3189-96.

4. King LE, Osati-Ashtiani F, Fraker PJ. Apoptosis plays a distinct role in the loss of precursor lymphocytes during zinc deficiency in mice. J Nutr. 2002 May;132(5):974-9.

5. Yarilin AA, Belyakov IM. Cytokines in the thymus: Production and biological effects. Curr Med Chem. 2004 Feb;11(4):447-64.

6. Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. Immunol Today. 1996 Mar;17(3):138-46.

7. Beck FW, Prasad AS, Kaplan J, Fitzgerald JT, Brewer GJ. Changes in cytokine production and T cell subpopulations in experimentally induced zinc-deficient humans. Am J Physiol. 1997 Jun;272(6 Pt 1):E1002-7.

8. Bao B, Prasad AS, Beck FW, Godmere M. Zinc modulates mRNA levels of cytokines. Am J Physiol Endocrinol Metab. 2003 Nov;285(5):E1095-102.

9. Guilbert JJ. The world health report 2002 - reducing risks, promoting healthy life. Educ Health (Abingdon). 2003 Jul;16(2):230.

10. Fraker PJ, King LE. Reprogramming of the immune system during zinc deficiency. Annu Rev Nutr. 2004;24:277-98.

11. Weight charts for select JAX mice strains [homepage on the Internet]. Bar Harbor, ME: The Jackson Laboratory. 2005 [cited August 7, 2006]. Available from: http://jaxmice.jax.org/info/weight/index.html.

12. Hosseinzadeh H, Goldschneider I. Recent thymic emigrants in the rat express a unique antigenic phenotype and undergo post-thymic maturation in peripheral lymphoid tissues. J Immunol. 1993 Mar 1;150(5):1670-9.

13. Panemangalore M, Bebe FN. Zinc diets: Deficiency and excess. In: Watson RR, editor. Trace elements in laboratory rodents. Boca Ratton, Florida: CRC press; 1997. p. 191-214.

14. Reeves PG, Nielsen FH, Fahey GC,Jr. AIN-93 purified diets for laboratory rodents: Final report of the american institute of nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr. 1993 Nov;123(11):1939-51.

15. Taylor CG, Bettger WJ, Bray TM. Effect of dietary zinc or copper deficiency on the primary free radical defense system in rats. J Nutr. 1988 May;118(5):613-21.

16. Institute for laboratory animal research, editor. Nutrient requirements of laborartory animals. fourth edition ed. National Academies Press; 1995.

4. ZINC-DEFICIENT RATS HAVE FEWER RECENT THYMIC EMIGRANT ($CD90^+$) T-LYMPHOCYTES IN SPLEEN AND BLOOD

Reproduced with permission from

Hosea HJ, Rector ES, Taylor CG. Zinc-deficient rats have fewer recent thymic emigrant (CD90+) T-lymphocytes in spleen and blood. J Nutr. 2003 Dec;133(12):4239-42.

^This research was presented in part at the Canadian Student Health Research Forum in Winnipeg, Manitoba, June 2004.

Hosea HJ, Rector ES, and Taylor CG. Elevated corticosterone concentrations do not explain fewer recent thymic emigrant ($CD90^+$) T-lymphocytes in zinc deficient rats. Nutrition, Lifestyle & Health Canadian Student Health Research Forum Symposium Program. 2004.

4.1 Abstract

It is hypothesized that increased expression of the signaling protein $p56^{lck}$ disrupts maturation of T-lymphocytes leading to the lymphopenia associated with dietary zinc deficiency and malnutrition. Our objective was to examine $p56^{lck}$ protein levels, flow cytometric markers of T-cell development (CD4, CD8, TCR$\alpha\beta$, TCR$\gamma\delta$, and CD90) and absolute cell numbers in thymus, spleen and blood of zinc-deficient (ZD), diet-restricted (DR) and control rats (CTL). Recent thymic emigrant ($CD90^+$) T-lymphocytes were also investigated after dietary repletion. $P56^{lck}$ protein levels were 2-fold greater in thymocytes than splenocytes, and ZD rats had a higher level of thymocyte $p56^{lck}$ than CTL. In the thymus and blood, the proportions of T-lymphocyte subpopulations ($CD4^-CD8^-$, $CD4^+CD8^+$ and $CD4^+CD8^-$ or $CD4^-CD8^+$) were unchanged, except for a higher percentage of TCR$\alpha\beta^+CD4^-CD8^+$ thymocytes in ZD animals. The 15-29% decrease in $CD90^+$ T-cells in the blood and spleens of ZD rats were reversed after dietary repletion for 7 and 23 days, respectively. In summary, T-cell numbers were proportional to thymus and spleen weights and unaltered in the blood, despite elevated thymocyte $p56^{lck}$ in ZD rats. In zinc deficiency, the decreased percentages of $CD90^+$ cells in the blood and spleen could adversely affect the T-cell repertoire.

4.2 Introduction

The lymphoid-specific protein tyrosine kinase $p56^{lck}$, which is principally expressed in T-lymphocytes, is essential for the development of thymocytes from $CD4^-CD8^-$ to $CD4^+CD8^+$ cells (1, 2). Abraham and colleagues (3) have also reported that in transgenic mice a twofold increase in $p56^{lck}$ expression delayed thymocyte development. Elevated expression of the zinc-finger protein $p56^{lck}$ has been demonstrated in T-lymphocytes from thymus and spleen of zinc-deficient and diet-restricted mice (4, 5). In dietary zinc deficiency and protein-energy malnutrition, reports of higher percentages of immature T-lymphocytes in the periphery (6-9) suggest that thymocyte development may be altered. Thus, we hypothesized that elevated $p56^{lck}$ expression may inhibit thymocyte maturation and this may contribute to the lymphopenia of zinc deficiency and protein-energy malnutrition.

The objective of the present investigation was to examine $p56^{lck}$ protein levels, flow cytometric markers of T-cell development (CD4, CD8, TCR$\alpha\beta$, TCR$\gamma\delta$, and CD90) and absolute T-cell numbers in thymus, spleen and blood of zinc-deficient (ZD) and diet-restricted (DR) rats after 3 weeks of deficiency. Recent thymic emigrant $CD90^+$ T-lymphocytes were also investigated after dietary repletion. The availability of fluorochrome-conjugated antibodies for the rat allowed us to investigate T-cell development in a rat model of zinc deficiency by flow cytometry. A peripheral lymphoid organ (spleen) and blood were included to determine if a disturbance in thymocyte development would be reflected in the periphery. The use of Flow-Count™ fluorospheres is an accepted

method for quantification of absolute cell numbers (10). Most T-cells express the T-cell receptor-αβ (TCRαβ) however, a small proportion express the TCRγδ. $p56^{lck}$ may also influence the development of TCRγδ cells (11). Thus, cell surface expression of both TCRαβ and TCRγδ was examined. To monitor the release of T-lymphocytes from the thymus into the periphery, recent thymic emigrants were identified by cell surface expression of CD90 (Thy1.1), which is present on newly released T-lymphocytes for 3-7 days (12).

4.3 Material and Methods

4.3.1 Animals and diets

Three week old Sprague Dawley rats (Charles River Laboratories, St. Constant, PQ) were acclimatized for 5 days and randomly assigned to the zinc deficient diet (ZD, <1 mg/kg zinc, *ad libitum*), or a nutritionally complete diet either *ad libitum* (CTL) or pair-fed to the ZD rats (diet-restricted group; DR) for 3 weeks (deficiency phase). During the repletion phase, rats were fed the control diet for 3, 7 or 23 days. The experimental diets (based on the AIN-93 diet), containing egg white and additional biotin (2 mg/kg diet) and potassium phosphate (5.4 g/kg diet for the growth formulation), and the controlled environmental conditions have been previously described (4). Body weights were determined weekly, and feed intake was determined daily. Animal care was provided in accordance with a protocol approved by the University of Manitoba Protocol Management and Review Committee.

4.3.2 Tissue collection

Rats were killed by CO_2 asphyxiation and decapitation. Trunk blood was collected in EDTA vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ). Thymi and spleens were removed aseptically, weighed and processed immediately. Femurs were removed, frozen at -20°C, and analyzed for zinc by atomic absorption spectrophotometery as previously described (4).

4.3.3 Outcome measurements

Determination of T-Lymphocyte subpopulations

Blood, or single cell suspensions from thymus and spleen (1×10^6 mononuclear cells; splenocytes separated by Lympholyte-Rat (Cedarlane, Hornby, ON)) were incubated with monoclonal antibodies (obtained from BD Pharminigen, Mississauga, ON) for TCR$\alpha\beta$ (phycoerythrin (PE) label, R73 clone), TCR$\gamma\delta$ (fluorescein isothiocyanate (FITC) label, V65 clone), CD90 (peridininchlorophyll-a protein (PerCP) label, Thy1.1, clone OX-7), CD4 (phycoerythrin-cyanine 5 (PC5) label, OX-35 clone), and CD8 (FITC label, G28 clone). The sample combinations for three-color analysis were tube1: TCR$\alpha\beta$, CD4, CD8 or their respective isotype controls, and tube 2: TCR$\alpha\beta$, TCR$\gamma\delta$, and CD90 or their respective isotype controls. Optilyse C (Beckman Coulter, Mississauga, ON) was used to lyse the red blood cells in blood. Flow-Count™ Fluorospheres (Beckman Coulter, Mississauga, ON) were added to tube 1 for thymus, spleen and blood to obtain absolute counts. Flow cytometry analysis was performed on a Beckman Coulter EPICS ALTRA (Beckman Coulter, Mississauga, ON) high-speed cell sorter using the EXPO32 MultiCOMP MFA software provided with the instrument.

Fluorochrome-isotype matched controls were prepared to assess autofluorescence and nonspecific binding, while single color samples were employed to adjust color compensation.

Western blotting of $p56^{lck}$

Cell lysates were prepared by resuspending thymocytes and splenocytes in buffer containing protease inhibitors as previously described (4). Protein concentration was determined using the Bicinchoninic acid Protein Assay (Sigma, St. Louis, MO). For Western blotting, cell lysates (20 μg protein per lane), molecular weight standard and positive control (Jurkat Cells Lysate, clone: Human T-cell leukemia, BD Pharmingen, Mississauga, ON) were separated by SDS-PAGE and transferred to nitrocellulose membrane (0.2 μm; BioRad, Hercules, CA) using previously published procedures (4). $P56^{lck}$ was detected using mouse anti-human lck (1:5000; clone 28, Transduction Laboratories, Lexington, KY), goat anti-mouse IgG horseradish peroxidase (1:1000) and Chemi Glow (Fisher, Whitby, ON) as the luminescent substrate. Arbitrary units for bands were determined using the FluorChem digital imaging system (Alpha Innotech Corporation, San Leandro, CA) and FluorChem software (version 2.0).

4.4 Statistical Analyses

Data were analyzed using the general linear models procedure (SAS software release 8.2, SAS Institute, Cary, NC). When necessary, data were normalized by log transformation for statistical analyses, but non-transformed means are reported. Significant differences among means were determined

using Duncan's multiple range test. Differences were considered significant at $P<0.05$.

4.5 Results

Zinc status and lymphoid organs. ZD consumed less feed, and weighed 49% less than CTL after 3 weeks of the dietary regimen (Table 4.6.1). ZD weighed 14% less than DR, despite similar feed consumption. Femur zinc concentrations in ZD and DR were 36% and 86%, respectively, of CTL. Thymus and spleen weights were lower in ZD compared to DR, and lymphoid organ weights were lower in DR compared to CTL. When lymphoid organ weight was corrected for body weight, there were no differences among the dietary treatment groups. T-cell numbers were proportional to thymus and spleen weights, and unaltered in blood.

$P56^{lck}$. Thymocytes had 2-fold greater $p56^{lck}$ protein levels than splenocytes (Figure 4.7.1). There were no differences in $p56^{lck}$ protein levels in splenocytes. However, the thymocytes from ZD expressed a higher level of $p56^{lck}$ compared to CTL.

T-Lymphocyte subpopulations. Cells were triple-labeled to differentiate T-cell maturation in the thymus ($TCR\alpha\beta^{-}CD4^{-}CD8^{-} \rightarrow TCR\alpha\beta^{-}CD4^{-}CD8^{+} \rightarrow TCR\alpha\beta^{-}CD4^{+}CD8^{+} \rightarrow TCR\alpha\beta^{+}CD4^{+}CD8^{+} \rightarrow TCR\alpha\beta^{+}CD4^{+}CD8^{-}$ or $TCR\alpha\beta^{+}CD4^{-}CD8^{+}$) and to identify T-lymphocyte subpopulations in the periphery. In the thymus, ZD had a higher percentage of $CD4^{-}CD8^{+}$ cells (gated on $TCR\alpha\beta^{+}$) compared to CTL (6.82±0.47% vs 4.82±0.45%, respectively). Otherwise, there were no differences

in the percentages of T-lymphocyte cell subpopulations in the thymus, spleen and blood (data not shown). Very few cells in the thymus, spleen and blood were TCR$\gamma\delta^+$ (0.34±0.02%, 1.69±0.10% and 0.89±0.05%, respectively) and no differences were observed due to dietary treatment (data not shown).

Recent thymic emigrants ($CD90^+$). In the thymus, there were no differences in the percentages of TCR$\alpha\beta^+$ cells that expressed CD90 among dietary treatment groups (Figure 4.7.2a). However, ZD had lower percentages of TCR$\alpha\beta^+$ cells that expressed CD90 in the spleen and blood compared to DR and CTL at the end of the deficiency phase (Figures 4.7.2b and 4.7.2c). In the blood and spleen, the percentages of TCR$\alpha\beta^+$ that expressed $CD90^+$ cells were similar in all groups after dietary repletion for 7 and 23 days, respectively.

4.6 Tables

Table 4.6.1. Body weight, femur zinc, lymphoid organ weight and cell numbers in zinc-deficient and diet-restricted rats[1]

	Dietary Group[2]		
	ZD	DR	CTL
Body weight, g	146.7 ± 2.1[c]	169.6 ± 5.2[b]	289.4 ± 5.8[a]
Total feed intake, g	204.7 ± 6.9[b]	213.2 ± 6.3[b]	427.1 ± 8.2[a]
Femur zinc, μmole zinc/g dry bone	1.64 ± 0.06[c]	4.50 ± 0.16[b]	5.26 ± 0.19[a]
Thymus weight, mg	396.1 ± 19.8[c]	512.9 ± 47.9[b]	896.4 ± 58.4[a]
Thymus/Body weight, %	0.27 ± 0.01	0.30 ± 0.02	0.31 ± 0.02
Thymic T-lymphocytes[3], cells/g thymus ($x10^8$)	742 ± 132	695 ± 109	509 ± 89
Spleen weight, mg	336.3 ± 22.9[c]	404.8 ± 23.8[b]	742.6 ± 36.6[a]
Spleen/Body weight, %	0.23 ± 0.01	0.24 ± 0.01	0.25 ± 0.01
Splenic T-lymphocytes[3], cells/g spleen ($x10^8$)	42.4 ± 5.4	54.7 ± 4.0	46.5 ± 6.2
Blood T-lymphocytes[3], cells/μL blood ($x10^6$)	4123 ± 592	5077 ± 285	4177 ± 428

[1]Values are means ± SEM, n=8. Different superscript letters indicate significant differences among dietary groups, $P < 0.05$

[2]ZD=zinc-deficient group; DR=diet-restricted group; CTL=control group

[3]T-lymphocytes ($TCR\alpha\beta^+$) were identified by flow cytometry, and absolute cell numbers (per g thymus or spleen, or per μL blood) were calculated using Flow-Count ™ Fluorospheres.

4.7 Figures

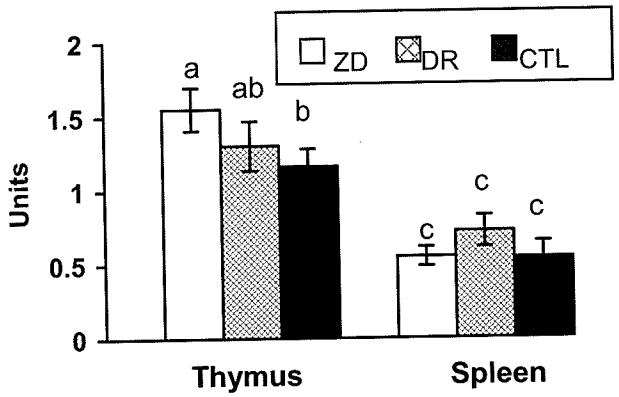


Figure 4.7.1. $p56^{lck}$ protein levels in thymocytes and splenocytes of zinc-deficient and diet-restricted rats. Values are means ± standard error for n=8. Different superscript letters indicate statistical differences ($p<0.05$). Abbreviations: ZD=zinc-deficient group; DR=diet-restricted group; CTL=control group.

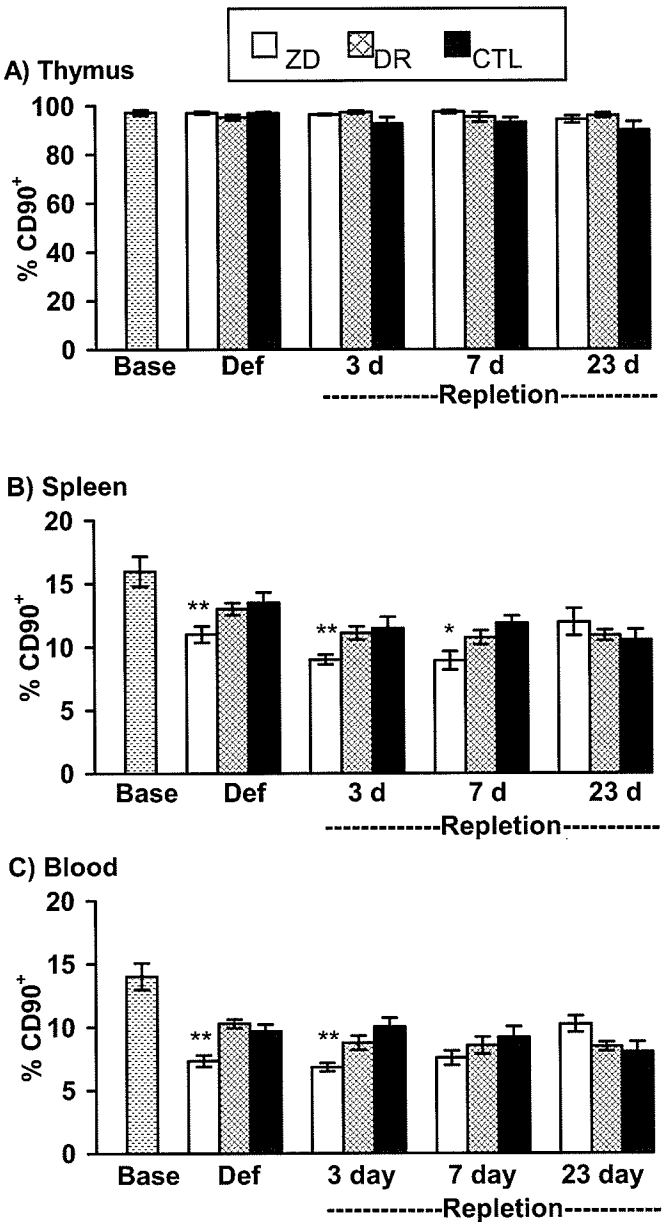


Figure 4.7.2. Flow cytometric analysis of CD90$^+$ T-lymphocytes (gated on TCRαβ$^+$ cells) in the thymus (A), spleen (B) and blood (C) of zinc-deficient and diet-restricted rats. Values are means ± standard error for n=8, except n=6 for 23 day repletion. Statistical differences ($P < 0.05$) among groups (ZD, DR and CTL) at each time point are indicated by asterisks: * indicates ZD is significantly different from CTL, and ** indicates ZD is significantly different from both DR and CTL. The Baseline group is a reference for 3 wk old rats at the beginning of the study. Abbreviations: ZD=zinc-deficient group; DR=diet-restricted group; CTL=control group; Base=Baseline group; Def=Deficiency phase.

4.8 Discussion

The major finding of the present study was that dietary zinc deficiency and diet restriction in growing rats did not alter thymocyte maturation based on TCR$\alpha\beta$, CD4 and CD8 flow cytometric markers, however, there was a reduced proportion of TCR$\alpha\beta^+$ cells that expressed CD90 (recent thymic emigrants) in the blood and spleen of ZD rats (Figure 4.7.2). During repletion with the control diet, the proportion of TCR$\alpha\beta^+$ cells that expressed CD90 in ZD rats recovered to control levels by 7 days in the blood and by 23 days in the spleen. In the thymus, there were no corresponding changes in the proportion of TCR$\alpha\beta^+$ cells that expressed CD90 due to dietary treatment.

CD90 (Thy1.1) is a cell surface marker that has been used to characterize thymocyte maturity and post-thymic maturation in rats during growth and in disease models (12, 13). Results from thymectomized rats indicate that approximately 80% of all circulating $CD90^+$ T-cells had emigrated from the thymus 3 days previously and that these cells were $CD90^-$ within 7 days (12). The age-related decline in the proportion of $CD90^+$ T-cells in the CTL rats between 3 and 9 weeks of age may be a reflection of reduced thymic replenishment of peripheral T-cells after the neonatal stage (14). The reduced proportion of recent thymic emigrant cells in ZD rats could be due to fewer T-cells released from the thymus, accelerated post-thymic maturation of $CD90^+$ cells, or fewer TCR$\alpha\beta^+$ cells that express CD90 surviving in the periphery. Greater susceptibility of $CD90^+$ cells to apoptosis has been reported in the diabetes-prone BB rat (13) and this needs to be experimentally tested in dietary zinc

deficiency. Furthermore, $p56^{lck}$ plays a role in signaling for apoptosis (15) and it is possible that the higher levels of $p56^{lck}$ in thymocytes of ZD rats (Figure 4.7.1) may predispose newly released $CD90^+$ T-lymphocytes to apoptosis in the periphery. However, the reduction in $TCR\alpha\beta^+$ cells that expressed $CD90^+$ in blood and spleen of ZD rats (Figure 4.7.2) did not lead to a change in T-cell numbers per gram spleen or µL blood. It appears that T-cell number and lymphoid organ weight (Table 4.6.1) are maintained proportional to animal size, regardless of nutritional state, in the growing rat. Although T-cell numbers per gram spleen or µL blood were not changed, it is possible that the altered proportion of recent thymic emigrant cells in zinc deficiency could adversely affect the T-cell repertoire of the animal over time (16, 17). In experimental zinc restriction in humans, there was a trend (P=0.077, n=5) towards a lower ratio of $CD4^+CD45RA^+$ (naïve) to $CD4^+CD45RO^+$ (memory) cells (18), supporting further investigation of the T-cell repertoire in zinc deficiency.

$P56^{lck}$ in thymic T-lymphocytes is essential for the maturation of T-cells from $CD4^-CD8^-$ to $CD4^+CD8^+$ (1, 2) and augmented expression of $p56^{lck}$ is associated with delayed thymocyte development (3). In the present experiment, there was no change in the percentages of $CD4^-CD8^-$ and $CD4^+CD8^+$ thymocytes in the ZD rats, inferring that $p56^{lck}$ protein was functional and that elevated $p56^{lck}$ levels were not disrupting thymic T-cell maturation. Thymic $TCR\alpha\beta^+CD4^+CD8^-$ cells were not altered by diet; however, ZD rats had a higher proportion of $TCR\alpha\beta^+CD4^-CD8^+$ cells in the thymus compared to CTL but not DR. A possible explanation is that strong TCR signals initiated by $p56^{lck}$ promote CD8

differentiation in rats and CD4 differentiation in mice (16). $P56^{lck}$ protein levels were elevated in thymus of ZD rats, but not spleen, compared to CTL but not DR (Figure 4.7.1). Elevated $p56^{lck}$ has been previously reported in thymocytes (5) and splenic T-lymphocytes (4) from zinc deficient mice, but comparison with diet-restricted mice (4) indicates that malnutrition also influences $p56^{lck}$ levels. In our previous study, splenic T-lymphocytes were isolated on immunocolumns, whereas mononuclear cells were used for Western blotting in the present investigation. Thus, the 2-fold greater expression of $p56^{lck}$ in the thymus compared to spleen (Figure 4.7.1) reflects the greater proportion of T-cells in the thymus.

Despite the severity of the zinc deficiency and diet restriction, there was no evidence of thymic or splenic atrophy relative to body weight or T-cell lymphopenia relative to organ weight or per μL blood in growing rats (Table 4.6.1) when absolute counts were determined using flow cytometry and Flow-Count™ Fluorospheres. In contrast, the young adult mouse model of dietary zinc deficiency is characterized by thymic atrophy, T-cell lymphopenia and significant changes in the phenotypic distribution of thymic T-cells when zinc-deficient mice weighed 30% less than zinc-adequate mice (19, 20). In adult zinc-deficient mice, there is a substantial reduction (38%) in the percentage of thymic $CD4^+CD8^+$ cells and a greater proportion of thymic $CD4^-CD8^-$, $CD4^+CD8^-$, and $CD4^-CD8^+$ (20). Thymic atrophy and T-cell lymphopenia have been reported in rodents and humans with protein-energy malnutrition (8, 21), but thymocyte subpopulations have not been characterized by flow cytometry. The present experiment

indicates that the young rat is able to maintain T-cell numbers proportional to their size, and that zinc deficiency and diet-restriction do not impair thymocyte maturation. Further research is required on nutritional deficiencies and the immune system in both growing and adult models as the implications for detection and therapeutic strategies may be dependent upon age and maturity of the immune system.

In summary, thymocyte $p56^{lck}$ was elevated in zinc deficiency, however, maturation of thymocytes from $CD4^{-}CD8^{-}$ to $CD4^{+}CD8^{+}$ cells was not altered in ZD and DR rats. In the growing rat, there was no evidence of lymphoid organ atrophy or T-cell lymphopenia in thymus, spleen or blood relative to organ weight or µL blood. The reduced percentages of $TCR\alpha\beta^{+}$ cells that express CD90 in the blood and spleen of ZD rats could adversely affect T-cell repertoire, limiting the recognition of foreign antigens. Future studies need to continue addressing the interactions among nutrition, age, maturity of the immune system and species differences.

4.9 Literature Cited

1. Molina TJ, Kishihara K, Siderovski DP, van Ewijk W, Narendran A, Timms E, Wakeham A, Paige CJ, Hartmann KU, Veillette A. Profound block in thymocyte development in mice lacking p56lck. Nature. 1992 May 14;357(6374):161-4.

2. Levin SD, Anderson SJ, Forbush KA, Perlmutter RM. A dominant-negative transgene defines a role for p56lck in thymopoiesis. EMBO J. 1993 Apr;12(4):1671-80.

3. Abraham KM, Levin SD, Marth JD, Forbush KA, Perlmutter RM. Delayed thymocyte development induced by augmented expression of p56lck. J Exp Med. 1991 Jun 1;173(6):1421-32.

4. Lepage LM, Giesbrecht JA, Taylor CG. Expression of T lymphocyte p56(lck), a zinc-finger signal transduction protein, is elevated by dietary zinc deficiency and diet restriction in mice. J Nutr. 1999 Mar;129(3):620-7.

5. Moore JB, Blanchard RK, McCormack WT, Cousins RJ. cDNA array analysis identifies thymic LCK as upregulated in moderate murine zinc deficiency before T-lymphocyte population changes. J Nutr. 2001 Dec;131(12):3189-96.

6. Chandra RK. T and B lymphocyte subpopulations and leukocyte terminal deoxynucleotidyl-transferase in energy-protein undernutrition. Acta Paediatr Scand. 1979 Nov;68(6):841-5.

7. Nash L, Iwata T, Fernandes G, Good RA, Incefy GS. Effect of zinc deficiency on autologous rosette-forming cells. Cell Immunol. 1979 Nov;48(1):238-43.

8. Parent G, Chevalier P, Zalles L, Sevilla R, Bustos M, Dhenin JM, Jambon B. In vitro lymphocyte-differentiating effects of thymulin (zn-FTS) on lymphocyte subpopulations of severely malnourished children. Am J Clin Nutr. 1994 Aug;60(2):274-8.

9. Bossuyt PJ, Rector ES, Taylor CG. Dietary zinc deficiency and protein-energy malnutrition decrease in vitro T lymphocyte cell cycle progression. J Nutr Immunol. 2001;5:71-83.

10. Bergeron M, Faucher S, Ding T, Phaneuf S, Mandy F. Evaluation of a universal template for single-platform absolute T-lymphocyte subset enumeration. Cytometry. 2002 Apr 15;50(2):62-8.

11. Fujise S, Matsuzaki G, Kishihara K, Kadena T, Molina T, Nomoto K. The role of p56lck in the development of gamma delta T cells and their function during an infection by listeria monocytogenes. J Immunol. 1996 Jul 1;157(1):247-54.

12. Hosseinzadeh H, Goldschneider I. Recent thymic emigrants in the rat express a unique antigenic phenotype and undergo post-thymic maturation in peripheral lymphoid tissues. J Immunol. 1993 Mar 1;150(5):1670-9.

13. Iwakoshi NN, Goldschneider I, Tausche F, Mordes JP, Rossini AA, Greiner DL. High frequency apoptosis of recent thymic emigrants in the liver of

lymphopenic diabetes-prone BioBreeding rats. J Immunol. 1998 Jun 15;160(12):5838-50.

14. Kendall MD. Functional anatomy of the thymic microenvironment. J Anat. 1991 Aug;177:1-29.

15. Di Somma MM, Nuti S, Telford JL, Baldari CT. p56lck plays a key role in transducing apoptotic signals in T cells. FEBS Lett. 1995 Apr 17;363(1-2):101-4.

16. Hunig T, Torres-Nagel N, Mehling B, Park HJ, Herrmann T. Thymic development and repertoire selection: The rat perspective. Immunol Rev. 2001 Dec;184:7-19.

17. Woodward B. Apoptotic loss of thymic lymphocytes in acute murine zinc deficiency. J Nutr. 2003 Mar;133(3):814; author reply 815.

18. Beck FW, Prasad AS, Kaplan J, Fitzgerald JT, Brewer GJ. Changes in cytokine production and T cell subpopulations in experimentally induced zinc-deficient humans. Am J Physiol. 1997 Jun;272(6 Pt 1):E1002-7.

19. Fraker PJ, King LE, Laakko T, Vollmer TL. The dynamic link between the integrity of the immune system and zinc status. J Nutr. 2000 May;130(5S Suppl):1399S-406S.

20. King LE, Osati-Ashtiani F, Fraker PJ. Apoptosis plays a distinct role in the loss of precursor lymphocytes during zinc deficiency in mice. J Nutr. 2002 May;132(5):974-9.

21. Slobodianik NH, Cosarinsky RC, Langini SH, Roux ME, Rio ME, Sanahuja JC. Effect of severe protein deficiency on surface and intracellular markers of growing rats' lymphoid organs. Nutr Rep Int. 1984;29:957-64.

5. DIETARY REPLETION CAN REPLENISH REDUCED T-CELL SUBSET NUMBERS AND LYMPHOID ORGAN WEIGHT IN ZINC-DEFICIENT AND ENERGY-RESTRICTED RATS

Reproduced with permission from

Hosea HJ, Rector ES, Taylor CG. Dietary repletion can replenish reduced T-cell subset numbers and lymphoid organ weight in zinc-deficient and energy-restricted rats. B J Nutr. 2004 May;91(5):741-7.

^This research was presented in part at the Dietitians of Canada's Annual Conference in Winnipeg, Manitoba, June 2001 and Experimental Biology in New Orleans, Louisiana, April 2002.

Hosea HJ, and Taylor CG. Effects of dietary zinc repletion on lymphoid tissue and lymphocyte number. Poster presentation, Dietitians of Canada's annual Professional Conference, June 2001. Cdn J Diet Prac Res. 2001; 62(2):114.

Hosea HJ, and Taylor CG. Lymphopenia in zinc deficient rats is associated with reduced, not elevated, serum corticosterone concentrations. Poster presentation and participant in the Nutritional Immunology Graduate Student Poster Competition, Experimental Biology, April 2002. FASEB J. 2002; 16(5): A620-621.

5.1 Abstract

The objective of the present study was to investigate the time course for recovery of lymphoid tissue and T-cell subset numbers when Zinc-deficient (ZD) or energy-restricted (ER) rats were repleted with control diet; in a second experiment, the link between the stress axis and lymphoid organs was explored. During the deficiency phase, rats were fed a ZD (<1 mg zinc/kg) or control diet (30 mg zinc/kg, nutritionally complete) either as pair-fed controls (ER) or *ad libitum*-fed controls (CTL) for 3 weeks. During the repletion phase, all rats were fed control diet *ad libitum* for 3, 7 or 23 days. After the deficiency phase, ZD and ER had lower T-cell subset numbers in the thymus compared to CTL, and ZD had reduced T-cell subset numbers in the spleen compared to both ER and CTL. T-cell subset numbers and lymphoid organ weights recovered from dietary zinc deficiency and energy restriction by 7 days of repletion (except 23 days for thymus weight in ZD) while body weight requires more than 23 days for recovery. At the end of the deficiency phase, ZD and ER had higher circulating corticosterone concentrations compared to CTL; plasma TNF-α was not detectable, and there were no differences in plasma haptoglobin, an acute phase protein. In conclusion, zinc deficiency and energy restriction elevated circulating corticosterone and reduced T-cell subset numbers in thymus and spleen of growing rats. Repletion with a nutritionally complete diet allowed recovery of T-cell subset numbers and lymphoid organ weight.

5.2 Introduction

All cells in the body require nutrients to function properly, and a deficiency in any of these required nutrients can cause immune function to be compromised. Both dietary zinc deficiency and energy malnutrition in mice are characterized by reduced growth, atrophy of lymphoid tissue, reduced lymphocyte numbers, and increased susceptibility to infection (1, 2). High concentrations of corticosterone have been shown to increase apoptosis *in vitro* in lymphocytes from rats (3); the decline in the proportion of $CD4^+CD8^+$ pre-T cells in the thymus of zinc-deficient mice has been attributed to greater apoptosis in this T-cell subset due to elevated circulating corticosterone concentrations (4). It has been suggested that dietary zinc deficiency stimulates the hypothalamus-pituitary-adrenal stress axis, leading to increased plasma corticosterone levels, and this may explain the lymphopenia and thymic atrophy associated with dietary deficiencies (5). Tumor necrosis factor-α (TNF-α) stimulates the hypothalamus-pituitary-adrenal axis through several intermediates, including adrenocorticotropic hormone (ACTH) which increases the release of corticosterone from the adrenal glands (6). TNF-α and corticosterone act on the liver to increase the induction of acute-phase proteins such as haptoglobin (6). Thus, the stress axis involves interactions among many components, including ACTH, corticosterone, TNF-α, and haptoglobin.

There has been a considerable amount of research on the effects of dietary Zinc deficiency and protein-energy malnutrition on immune function; however, the role of nutrients in recovery of the immune system from nutritional

deprivation is important for development of nutritional therapies. Thus, our first objective was to investigate the time course for recovery of lymphoid tissue and T-cell subset numbers when Zinc-deficient or energy-restricted rats were repleted with a nutritionally complete control diet. In a second experiment, the potential link between markers of the stress axis (ACTH, corticosterone, TNF-α and haptoglobin) and atrophy of the lymphoid organs in the dietary deficiencies was explored. To separate the effects of Zinc deficiency from energy malnutrition, an energy-restricted group (pair-fed to the Zinc-deficient rats) was included. Three time points were chosen for dietary repletion: 3 days to identify any rapid changes (lymphocytes proliferate quickly), 7 days based on recovery of thymus and spleen weights in adult Zinc-deficient or protein-malnourished mice (7, 8), and 23 days based on the timeframe for murine T-lymphocyte maturation (9). The growing rat model represents an age group that is at risk for Zinc deficiency and energy malnutrition (10). The commercial availability of antibodies for the rat presents new opportunities to investigate immune function using rat models in which nutritional interventions have been extensively characterized.

5.3 Material and Methods

5.3.1 Animals and diets

Experiment 1: Ninety-eight, three week old male Sprague Dawley rats (Charles River Laboratories, St. Constant, PQ) were acclimatized for 5 days; they were randomly assigned to the baseline group (n=8) or were fed a Zinc-deficient diet *ad libitum* (ZD group; <1 mg zinc/kg; n=30), or a nutritionally complete control

diet (30 mg zinc/kg) either *ad libitum* (CTL; n=30) or pair-fed to the ZD group (ER; energy restricted; n=30) for 3 weeks (deficiency phase). At the end of the deficiency phase, 8 animals per dietary treatment group were killed and the remaining twenty-two rats per group began the repletion phase. During the repletion phase, rats were fed the control diet for 3 (eight per group), 7 (eight per group) or 23 (six per group) days. The experimental diets, containing egg white and additional biotin (2 mg/kg diet) and potassium phosphate (5.4 g/kg diet for the growth formulation), have been previously described by Lepage and colleagues (11). The Zinc content of the diets was verified by atomic absorption analysis. Care was taken to avoid Zinc recycling and contamination by housing the rats in stainless steel hanging cages with mesh bottoms, and providing distilled water in plastic bottles with stainless steel sipper tubes. The rats were maintained in an environment of controlled temperature (21-23°C), humidity (55%) and light cycle (14 h light/10 h dark). Body weights were determined weekly, and feed intake was determined daily. Animal care was provided in accordance with a protocol approved by the Local Animal Care Committee (University of Manitoba).

Experiment 2: To investigate the response of the stress axis, the animals and diets were as described above but confined to the deficiency phase only. The rats were handled by the same investigator on a daily basis during the acclimation and experimental period to minimize the effects of handling stress before phlebotomy (12).

5.3.2 Sample collection

At baseline, after the 3-week deficiency phase, and after 3, 7 or 23 days of repletion, rats were killed by CO_2 asphyxiation and decapitation between 08:00 and 09:00 hours. Trunk blood was collected in EDTA vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) or blood collection tubes, centrifuged to obtain plasma or serum, and stored at -80°C until analysis. Thymus and spleen were removed aseptically, weighed and processed immediately.

5.3.3 Outcome measurements

Zinc analysis

After obtaining wet and dry weights, thymus, spleen and diet samples were wet-ashed using trace-element grade nitric acid. After appropriate dilution of digests, zinc concentration was determined by atomic absorption spectroscopy using a Spectra AA-30 Spectrophotometer (Varian Canada, Georgetown, ON). Quality control was monitored using bovine liver standard reference material 1577b (U.S. Department of Commerce, National Institute of Standards and Technology, Gaithersburg, MD).

Determination of T-lymphocyte subpopulations

Single-cell suspensions were obtained by gently suspending the thymus and spleen in phosphate buffered saline supplemented with 1% bovine fetal calf serum (Gibco, Grand Island, NY) using a glass-glass tissue grinder (Koontes, Vineland, NJ). Cells remained intact as verified by trypan blue exclusion. Single cell suspensions from thymus and spleen (1×10^6 mononuclear cells; splenocytes separated by Lympholyte-Rat (Cedarlane, Hornby, ON)) were

incubated with monoclonal antibodies (obtained from BD Pharminigen, Mississauga, ON) for TCR$\alpha\beta$ (PE label, R73 clone), CD4 (PC5 label, OX-35 clone), and CD8 (FITC label, G28 clone). Flow-Count^{TM} Fluorospheres (Beckman Coulter, Mississauga, ON) were added to obtain absolute counts. Flow cytometry analysis was performed on a Beckman Coulter EPICS ALTRA (Beckman Coulter, Mississauga, ON) high-speed cell sorter using the EXPO32 MultiCOMP software provided with the instrument. Forward angle versus side scatter histograms were used to gate for single cells. Fluorochrome-isotype matched controls were prepared to assess autofluorescence and nonspecific binding, while samples stained individually with a single fluorochrome were employed to adjust color compensation. The data was collected in listmode format and the subsequent analysis based on 10 000 cells satisfying the light scatter gating criteria. Absolute counts of T-cell subsets were calculated based on the total number of cells counted, the total number of fluorospheres counted and the concentration of Flow-Count^{TM} Fluorospheres. Cells counts were corrected for the weight of the thymus or spleen used to prepare the cell suspensions.

Biochemical measurements

Corticosterone and ACTH concentrations were determined by radioimmunoassay kits (ICN Biomedicals, Inc., Costa Mesa, CA). Plasma TNF-α and haptoglobin concentrations were determined using an ELISA (Alpco Diagnostics, Whidham, NH; detection limit 15.6 pg/ml) and colorimetric assay

(Tridelta Development, Wicklow, Ireland), respectively. Samples were analyzed in duplicate and percent agreement was >85% (r^2>0.99 for standard curves).

5.4 Statistical Analysis

Differences among dietary treatment groups and over time were analyzed by one-way ANOVA using the general linear models procedure (Statistical Analysis Systems software release 8.2, SAS Institute, Cary, NC). When necessary, data were normalized by log transformation for statistical analyses, but non-transformed means are reported. Significant differences among means were determined using Duncan's New Multiple Range test. Differences were considered significant at $p<0.05$.

5.5 Results

ZD consumed less feed per day (Table 5.6.1), and weighed 49% less than CTL at the end of the deficiency phase (Figure 5.7.1a). For ZD and ER there was no difference in feed consumption during the deficiency phase, but ZD weighed 14% less than ER. The high feed efficiency ratio reveals that ZD needed to consume more feed to gain the same weight as the other groups during the deficiency phase. During the first 3 days of repletion with the control diet, the feed intake of ZD and ER rats increased 88% and 123%, respectively, while CTL increased feed intake by only 15%. During repletion, ZD and ER also had greater rate of weight gain than CTL. Throughout the repletion phase, both ZD and ER had lower feed efficiency ratios than CTL, indicating that ZD and ER

were able to gain more weight with less feed compared to CTL during the repletion phase.

The lymphoid organ to body weight ratios were highest at baseline and decreased with age in all groups (Figure 5.7.1c & 5.7.1e). There were no differences among groups in either thymus to body weight or spleen to body weight ratios at the end of the deficiency phase. Both thymus and spleen weights were lower in ZD and ER compared to CTL at the end of the deficiency phase, and ZD was lower than ER (Figure 5.7.1b & 5.7.1d). During the deficiency phase, the lymphoid organ weights of ZD decreased 20% compared to baseline, and there was no change in ER, while thymus and spleen weights of CTL increased 81% and 78%, respectively, compared to baseline. In ZD, spleen weight recovered to CTL values by 7 days and thymus weight recovered by 23 days of repletion. In ER, both thymus and spleen weights recovered to control levels by 7 days of repletion. There were no differences in thymus or spleen zinc concentrations throughout the study (data not shown).

There was no difference among dietary treatment groups in the absolute number per organ of the most immature thymic cells ($TCR\alpha\beta^{-}CD4^{-}CD8^{-}$; results not shown). At the end of the deficiency phase, ZD and ER rats had 35-52% fewer thymic pre-T cells ($TCR\alpha\beta^{-}CD4^{+}CD8^{+}$and $TCR\alpha\beta^{+}CD4^{+}CD8^{+}$) compared to CTL, and ZD had fewer thymic $TCR\alpha\beta^{+}CD4^{+}CD8^{-}$ (helper) cells compared to CTL (Figure 5.7.2a-d). There were no differences in thymic T-cell subset numbers among dietary treatment groups after 7 days of repletion. ZD rats had 40-63% fewer splenic $TCR\alpha\beta^{+}CD4^{+}CD8^{-}$ (helper) and $TCR\alpha\beta^{+}CD4^{-}CD8^{+}$

(cytolytic) cells (Figure 5.7.2e & 2f) compared to both ER and CTL at the end of the deficiency phase, but not at any other time point. ER had 39% fewer splenic helper T-cells compared to CTL at the end of the deficiency phase, but recovered to CTL levels after 3 days of repletion. There were no differences in the number of T-cell subsets among dietary treatment groups when corrected for lymphoid organ weight (data not shown).

Adrenal weight to body weight ratios were highest at baseline (Figure 5.7.3). At the end of the deficiency phase, ZD and ER had 65% and 34%, respectively, higher adrenal gland weight to body weight ratios compared to CTL, and ZD was 23% higher than ER. The adrenal to body weight ratio was not different from CTL after 3 days of repletion for ER and after 7 days of repletion for ZD.

Markers of the stress axis were determined in a separate group of rats where special care was taken to minimize environmental stressors. ZD and ER had 338-527% higher serum corticosterone concentrations compared to CTL (Table 5.6.2). There were no differences among dietary treatment groups for plasma ACTH and haptoglobin concentrations, and TNF-α was not detectable in the plasma (results not shown).

5.6 Tables

Table 5.6.1. Effects of zinc deficiency and energy restriction followed by repletion on feed efficiency in growing rats.[†]

		Dietary Group[§]					
	Time[‡]	ZD		ER		CTL	
		Mean	SE	Mean	SE	Mean	SE
Feed intake, g/day	Deficiency	9.8^{b*}	0.3	10.2^{b*}	0.3	20.3^{b}	0.4
	3 Day Repletion	18.4^{a*}	0.9	22.7^{a}	0.6	23.3^{a}	0.7
	7 Day Repletion	20.0^{a**}	0.7	23.0^{a}	0.9	23.9^{a}	1.2
	23 Day Repletion	20.2^{a}	0.8	22.1^{a}	0.7	22.5^{ab}	1.0
Weight gain[‖] g/100 g bwt/d	Deficiency	1.2^{c**}	0.1	1.9^{d*}	0.2	4.2^{a}	0.1
	3 Day Repletion	6.8^{a**}	0.3	8.2^{a*}	0.3	2.6^{b}	0.1
	7 Day Repletion	6.1^{a*}	0.3	6.2^{b*}	0.1	2.3^{b}	0.2
	23 Day Repletion	3.5^{b*}	0.1	3.2^{c*}	0.1	1.6^{c}	0.1
Feed efficiency ratio[¶]	Deficiency	2.6^{a**}	0.1	2.1^{b}	0.1	1.9^{c}	0.1
	3 Day Repletion	1.7^{b*}	0.1	1.5^{d*}	0.1	3.1^{b}	0.2
	7 Day Repletion	1.8^{b*}	0.1	1.8^{c*}	0.1	3.9^{ab}	0.8
	23 Day Repletion	2.5^{a*}	0.1	2.6^{a*}	0.1	4.3^{a}	0.3

[†] Values are means ± standard error for n=8 (baseline, deficiency, 3 day and 7 day repletion) and n=6 (23 day repletion). Abbreviations: ZD=zinc-deficient group; ER=energy-restricted group; CTL=control group.

[‡] Different superscript letters indicate statistical differences ($p<0.05$) over time.

[§] Statistical differences ($p<0.05$) among groups (ZD, ER and CTL) at each time point are indicated by asterisks: ** indicates ZD is significantly different from both ER and CTL and * indicates ZD or ER are significantly different from CTL.

[‖] Rate of weight gain = [(final body weight (g)-initial body weight (g))/average weight (g)]/days.

[¶] Feed efficiency ratio = total feed intake (g)/total weight gained (g).

Table 5.6.2. Effects of zinc deficiency and energy restriction on circulating markers of the stress axis.†

	Dietary Group‡					
	ZD		ER		CTL	
	Mean	SE	Mean	SE	Mean	SE
Serum Corticosterone, nmol/L	329*	79	508*	131	9.46	0.98
Plasma ACTH, pg/mL	605	110	727	96	548	91
Plasma haptoglobin, mg/mL	0.69	0.05	0.65	0.05	0.52	0.06

† Values are means ± standard error of means, n=9.

‡ Statistical differences ($p<0.05$) among groups are indicated by asterisks: * indicates ZD or ER are significantly different from CTL, but not from each other.

Abbreviations: ZD=zinc-deficient group; ER=energy-restricted group; CTL=control group.

5.7 Figures

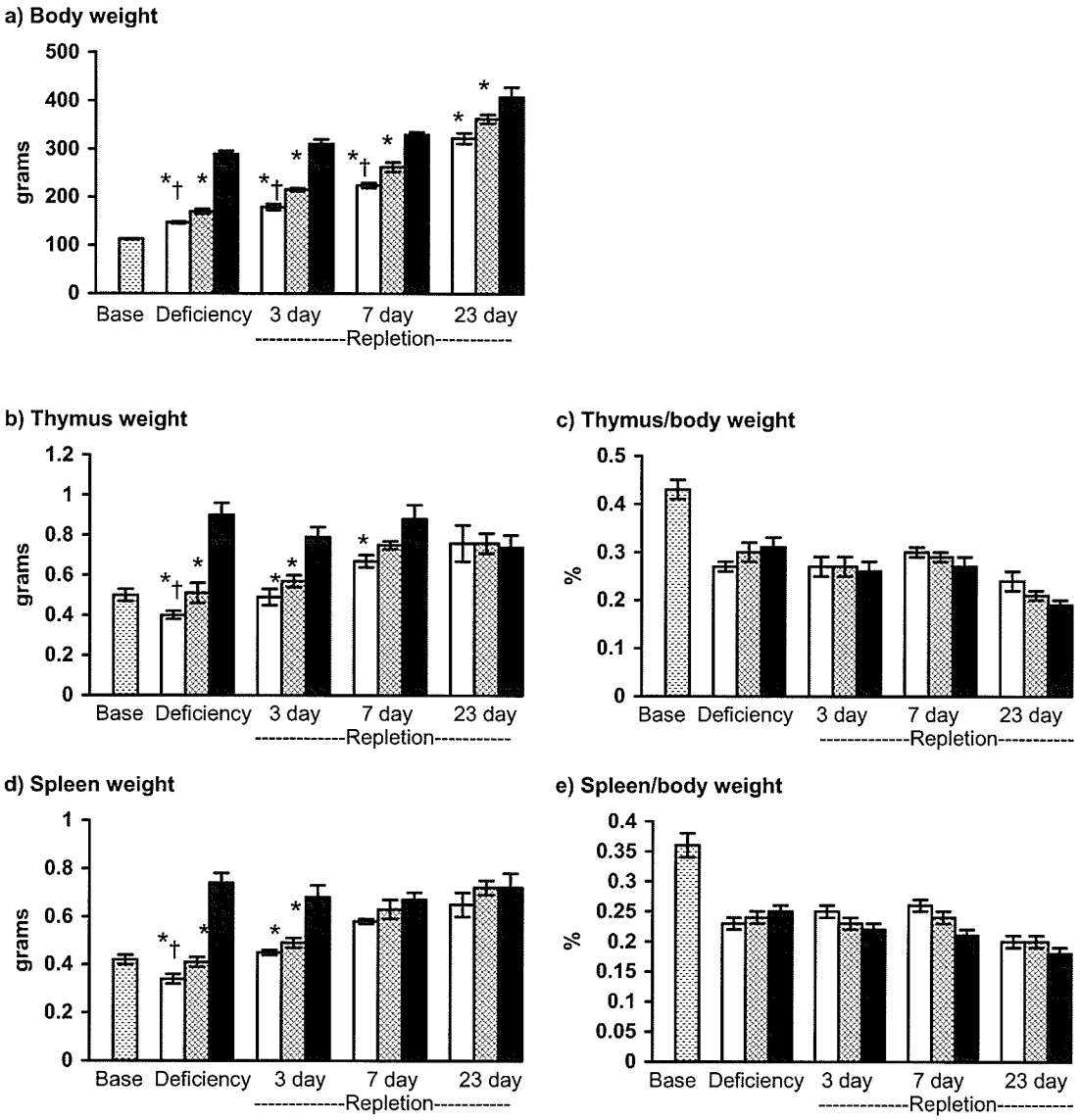


Figure 5.7.1. Effects of Zinc deficiency, energy restriction and repletion on body, thymus and spleen weights and ratios in growing rats. ☐ Zinc-deficient group; ☒ energy-restricted group; ■ control group. For details of diets and procedures, see Material and methods. Values are means ± standard errors shown by vertical bars (baseline, deficiency, 3 day and 7 day repletion: eight rats per group; 23 day repletion: six rats per group). Mean values were significantly different from those of the control group at each time point: *P<0.05. Mean values were significantly different from those of the energy-restricted group at each time point: †P<0.05.

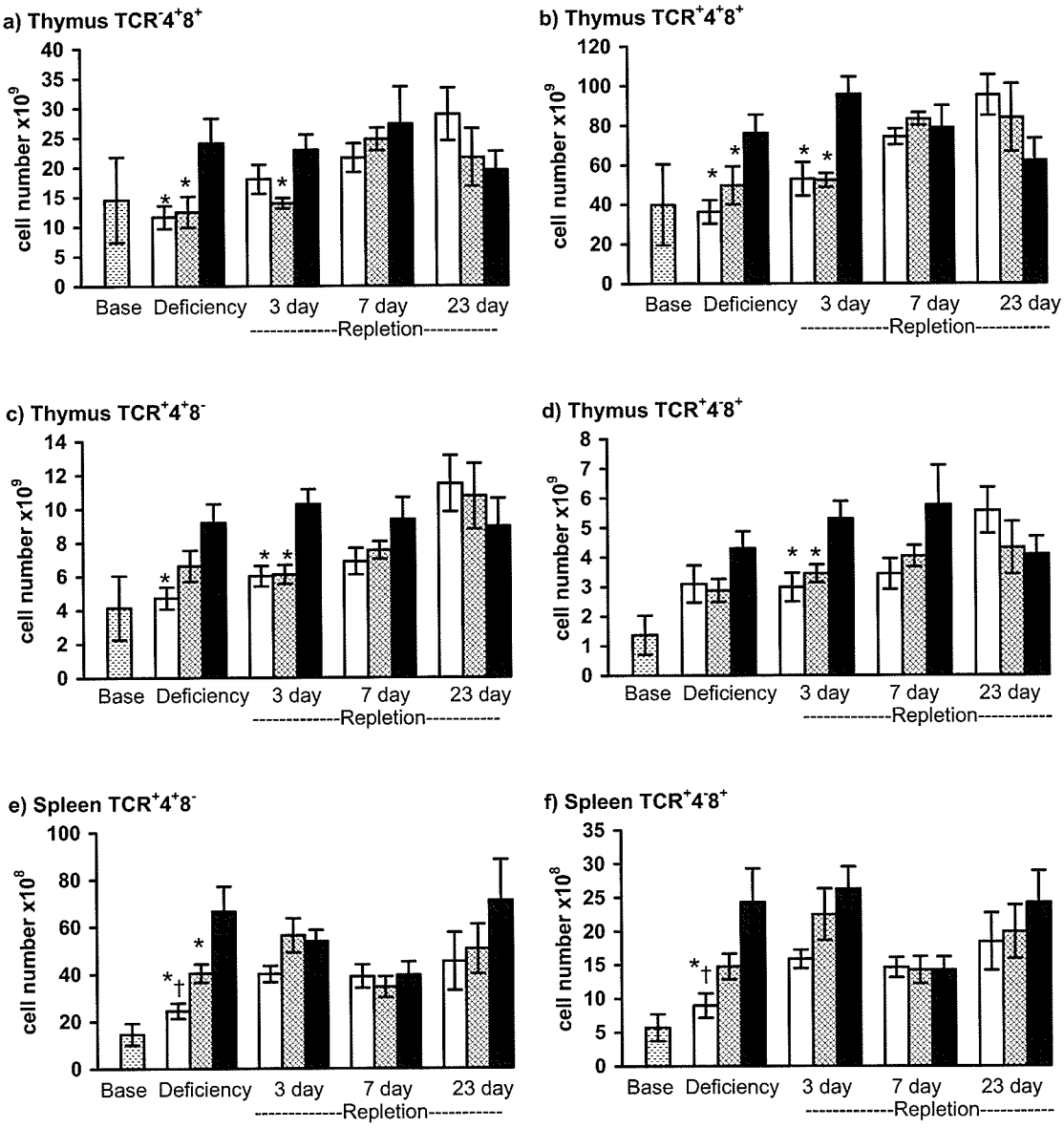


Figure 5.7.2. Effects of zinc deficiency, energy restriction and repletion on thymus and spleen T-cell subset numbers (per organ) in growing rats. ☐ Zinc-deficient group; ☒ energy-restricted group; ■ control group. For details of diets and procedures, see Material and methods. (a) Thymus TCRαβ$^-$CD4$^+$CD8$^+$; (b) thymus TCRαβ$^+$CD4$^+$CD8$^+$; (c) thymus TCRαβ$^+$CD4$^+$CD8$^-$; (d) thymus TCRαβ$^+$CD4$^-$CD8$^+$; (e) spleen TCRαβ$^+$CD4$^+$CD8$^-$; (f) spleen TCRαβ$^+$CD4$^-$CD8$^+$. Cells were triple-labeled to identify T-cells at various stages of maturation in the thymus. T-cell maturation from immature to mature is as follows: TCRαβ$^-$CD4$^-$CD8$^-$ (Pro-T cell) → TCRαβ$^-$CD4$^+$CD8$^+$ (Pre-T cell) → TCRαβ$^+$CD4$^+$CD8$^+$ (Pre-T cell) → TCRαβ$^+$CD4$^+$CD8$^-$

(helper T-cell) or $TCR\alpha\beta^{+}CD4^{-}CD8^{+}$ (cytolytic T-cell). Values are means ± standard errors shown by vertical bars (baseline, deficiency, 3 day and 7 day repletion: eight rats per group; 23 day repletion: six rats per group). Mean values were significantly different from those of the control group at each time point: *$P<0.05$. Mean values were significantly different from those of the energy-restricted group at each time point: †$P<0.05$.

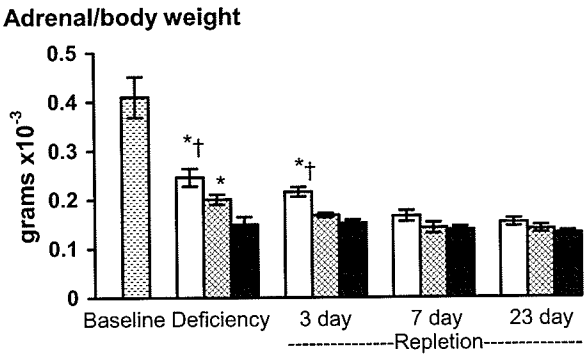


Figure 5.7.3. Effects of zinc deficiency, energy restriction and repletion on adrenal weight/body weight ratios in growing rats. ☐ Zinc-deficient group; ⊠ energy-restricted group; ■ control group. For details of diets and procedures, see Material and methods. Values are means ± standard errors shown by vertical bars (baseline, deficiency, 3 day and 7 day repletion: eight rats per group; 23 day repletion: six rats per group). Mean values were significantly different from those of the control group at each time point: *P<0.05. Mean values were significantly different from those of the energy-restricted group at each time point: †P<0.05.

5.8 Discussion

Both zinc deficiency and energy restriction in growing rats elevated circulating corticosterone and reduced thymic pre-T cell numbers, while zinc-deficient rats had fewer splenic helper and cytolytic T-cell numbers compared to energy-restricted and control rats. In addition, ZD had fewer thymic helper T-cells and ER had fewer splenic helper T-cells compared to CTL at the end of the deficiency phase. T-cell subset numbers and lymphoid organ weights recovered from dietary zinc deficiency and energy restriction after 7 days of repletion with a nutritionally complete diet, while body weight requires longer than 23 days to catch up to CTL in growing rats. Spleen weight and spleen T-cell subset numbers recovered faster than the same parameters in the thymus of ZD and ER rats. There appears to be a priority for recovery of lymphoid organs before body weight enabling the body to produce more T-lymphocytes and release them into circulation for immune defense while nutritional recovery is in progress.

In growing rats, reduced dietary intake of zinc or energy results in stunting malnutrition (Figure 5.7.1). As expected thymus and spleen weights were lower in ZD and ER at the end of the deficiency phase and this is similar to the adult mouse model (13-15). Lymphoid organ atrophy relative to body weight is present in the adult zinc-deficient mouse (11, 13, 15), a model of wasting malnutrition. However, in growing zinc-deficient rats, a model of stunting malnutrition, lymphoid organ weights relative to body weight were not different from ER or CTL (Figure 5.7.2; (16)). In growing zinc-deficient rats, the substantial (70%) reduction of femur zinc concentrations (17) indicates the severity of the zinc

deficiency, however, there was no thymic or splenic atrophy relative to body weight (Figure 5.7.1) which is used as an indicator of severe immunodeficiency (2). Others have reported reduced lymphocyte numbers in rodent models of zinc deficiency and protein energy malnutrition and there was a greater reduction of lymphocyte numbers in presence of wasting malnutrition compared to stunting malnutrition (15, 18). In the present study using flow cytometry and Flow Count™ Flurospheres, ZD and ER had lower thymus and spleen cell numbers (sum of subsets) compared to CTL, and ZD had fewer spleen TCR$\alpha\beta^+$ cell numbers compared to ER at the end of the deficiency phase (Figure 5.7.2). Cell numbers responded rapidly to repletion, recovering to CTL levels by 7 days in ER and ZD. T-cell maturation takes approximately 3 weeks in mice (9), thus, the increase in cell numbers reflects the ability of existing cells to replicate. Thymus and spleen cell numbers per gram of tissue were not different between ZD and ER in growing rats (present study) or in adult mice (11). Although ZD and ER rats maintain lymphoid cell numbers proportional to tissue weight and lymphoid organ weights proportional to body weight, the lower number of lymphocytes per animal may reduce the T-cell repertoire and may contribute to the greater susceptibility of deficient animals to infection (19).

In the present study, serum corticosterone concentrations were elevated in both ZD and ER compared to the CTL (Table 5.6.2). Others have reported that 3-6 week old mice fed zinc-deficient diet for 19-31 days have approximately 200% higher plasma corticosterone concentrations (determined by spectrofluorometric method) and a 33% larger adrenal gland weight to body

weight ratio compared to mice fed the zinc-adequate diet *ad libitum* (20). When male Sprague Dawley rats (150-160 g) were fed zinc-deficient diet for 40 days, serum corticosterone concentrations (determined by a radioimmunoassay kit from ICN) were 122% higher than rats fed the control diet *ad libitum* (21). Neither of these studies reported the circulating corticosterone concentrations in an energy-restricted group. The cytokine TNF-α and acute phase protein haptoglobin have been associated with corticosterone concentrations during inflammation (6), but plasma TNF-α was not detected and haptoglobin was unchanged by dietary treatment indicating the absence of infection these animals.

One of the hypothesizes for the low lymphoid organ cell numbers in zinc deficiency is that elevated corticosterone promotes apoptosis in lymphoid cells (5). King and colleagues (4) have reported elevated serum corticosterone and higher proportions of apoptosis in pre-T cells and a lower percentage of these cells in the thymus of zinc-deficient mice, whereas Moore and colleagues (22) found no changes in proportions of thymic T-cells in zinc-deficient mice. Both studies reported a similar reduction in serum zinc, however, there was no weight loss in the Moore and colleagues (22) study while zinc-deficient mice in the King and colleagues (4) study weighed 29% less than control mice. Although feed intake was similar (4), the specific role of zinc deficiency versus the role of weight loss was not addressed. In the growing rat model of zinc deficiency, we found no changes in the proportions of T-cell subsets in the thymus and spleen, except a higher proportion of thymic cytolytic T-cells in zinc-deficient rats (17). In the

present study, ZD and ER had reduced numbers of some T-cell subsets per organ, but there were no differences when T-cell subsets were expressed per gram of tissue. Both ZD and ER had elevated circulating corticosterone and reduced thymic pre-T cell numbers, but in the spleen, ZD had fewer helper and cytolytic T-cell numbers compared to ER and CTL. Although thymus and spleen zinc concentrations were unchanged, other variables in the periphery related to low zinc status may be contributing factors.

In summary, lymphoid cell numbers and lymphoid organ weights recover more rapidly from zinc deficiency and energy restriction in growing rats than body weight. Thus, the body is producing more T-lymphocytes and releasing them into circulation for immune defense while nutritional recovery is in progress. Future studies need to assess the functional recovery of the immune system, including resistance to infection.

5.9 Literature Cited

1. Woodward B. Protein, calories, and immune defenses. Nutr Rev. 1998 Jan;56(1 Pt 2):S84-92.

2. Fraker PJ, King LE, Laakko T, Vollmer TL. The dynamic link between the integrity of the immune system and zinc status. J Nutr. 2000 May;130(5S Suppl):1399S-406S.

3. Hughes FM,Jr, Cidlowski JA. Glucocorticoid-induced thymocyte apoptosis: Protease-dependent activation of cell shrinkage and DNA degradation. J Steroid Biochem Mol Biol. 1998 Apr;65(1-6):207-17.

4. King LE, Osati-Ashtiani F, Fraker PJ. Apoptosis plays a distinct role in the loss of precursor lymphocytes during zinc deficiency in mice. J Nutr. 2002 May;132(5):974-9.

5. Fraker PJ, Osati-Ashtiani F, Wagner MA, King LE. Possible roles for glucocorticoids and apoptosis in the suppression of lymphopoiesis during zinc deficiency: A review. J Am Coll Nutr. 1995 Feb;14(1):11-7.

6. Steel DM, Whitehead AS. The major acute phase reactants: C-reactive protein, serum amyloid P component and serum amyloid A protein. Immunol Today. 1994 Feb;15(2):81-8.

7. Fraker PJ, DePasquale-Jardieu P, Zwickl CM, Luecke RW. Regeneration of T-cell helper function in zinc-deficient adult mice. Proc Natl Acad Sci U S A. 1978 Nov;75(11):5660-4.

8. Taylor CG, Potter AJ, Rabinovitch PS. Splenocyte glutathione and CD3-mediated cell proliferation are reduced in mice fed a protein-deficient diet. J Nutr. 1997 Jan;127(1):44-50.

9. Sharon J. Basic immunology. Baltimore: Williams and Williams; 1998.

10. Briefel RR, Bialostosky K, Kennedy-Stephenson J, McDowell MA, Ervin RB, Wright JD. Zinc intake of the U.S. population: Findings from the third national health and nutrition examination survey, 1988-1994. J Nutr. 2000 May;130(5S Suppl):1367S-73S.

11. Lepage LM, Giesbrecht JA, Taylor CG. Expression of T lymphocyte p56(lck), a zinc-finger signal transduction protein, is elevated by dietary zinc deficiency and diet restriction in mice. J Nutr. 1999 Mar;129(3):620-7.

12. Shipp K, Woodward BD. A simple exsanguination method that minimizes acute pre-anesthesia stress in the mouse: Evidence based on serum corticosterone concentrations. Contemp Top Lab Anim Sci. 1998 Sep;37(5):73-7.

13. Fraker PJ, Haas SM, Luecke RW. Effect of zinc deficiency on the immune response of the young adult A/J mouse. J Nutr. 1977 Oct;107(10):1889-95.

14. Fraker PJ, Zwickl CM, Luecke RW. Delayed type hypersensitivity in zinc deficient adult mice: Impairment and restoration of responsivity to dinitrofluorobenzene. J Nutr. 1982 Feb;112(2):309-13.

15. Cook-Mills JM, Fraker PJ. Functional capacity of the residual lymphocytes from zinc-deficient adult mice. Br J Nutr. 1993 May;69(3):835-48.

16. Giugliano R, Millward DJ. Growth and zinc homeostasis in the severely zn-deficient rat. Br J Nutr. 1984 Nov;52(3):545-60.

17. Hosea HJ, Rector ES, Taylor CG. Zinc-deficient rats have fewer recent thymic emigrant (CD90+) T lymphocytes in spleen and blood. J Nutr. 2003 Dec;133(12):4239-42.

18. Bises G, Mengheri E, Gaetani S. T-lymphocyte subsets in zinc deficient and in protein malnourished rats. Nutr Rep Int. 1987;36:1371-8.

19. Woodward B. Apoptotic loss of thymic lymphocytes in acute murine zinc deficiency. J Nutr. 2003 Mar;133(3):814; author reply 815.

20. DePasquale-Jardieu P, Fraker PJ. The role of corticosterone in the loss in immune function in the zinc-deficient A/J mouse. J Nutr. 1979 Nov;109(11):1847-55.

21. Nobili F, Vignolini F, Figus E, Mengheri E. Treatment of rats with dexamethasone or thyroxine reverses zinc deficiency-induced intestinal damage. J Nutr. 1997 Sep;127(9):1807-13.

22. Moore JB, Blanchard RK, McCormack WT, Cousins RJ. cDNA array analysis identifies thymic LCK as upregulated in moderate murine zinc deficiency before T-lymphocyte population changes. J Nutr. 2001 Dec;131(12):3189-96.

6. AGE-RELATED CHANGES IN P56^{LCK} PROTEIN LEVELS AND PHENOTYPIC DISTRIBUTION OF T-LYMPHOCYTES IN YOUNG RATS

Reproduced with permission from

Hosea HJ, Rector ES, Taylor CG. Age-related changes in p56^{lck} protein levels and phenotypic distribution of T-lymphocytes in young rats. Clin Dev Immunol. 2005 March;12(1):75-84.

6.1 Abstract

$p56^{lck}$ is involved in the maturation of T-cells from double negative (DN) into double positive (DP) T-cells. The objective of this experiment was to determine changes in the levels of thymic and splenic T-cell $p56^{lck}$ using Western immunoblotting, along with the proportion and number of the T-cell subsets in the thymus, spleen and blood using flow cytometry in growing Sprague Dawley rats. Thymic $p56^{lck}$ protein levels were negatively correlated with age ($r= -0.42$, $p=0.04$) and positively correlated with age in the spleen ($r= 0.5$, $p=0.01$). Nine-week-old rats had a higher percentage of thymic DN and CD8 cells with fewer DP cells compared to younger rats. There were minor differences in the proportions of T-cell subsets in the spleen and blood. T-cell numbers remained proportional to body weight in the lymphoid organs; however, the lower absolute number of T-cells in the younger rats might indicate that they are less able to respond to antigens.

6.2 Introduction

The immune system is complex and is affected by numerous environmental and genetic factors. Inbred, transgenic and knock-out mouse strains have been invaluable tools for advancing our knowledge of the immune system (1-3). However, the rat is also an important model in experimental biology, and the emerging importance of the immune system in various disciplines and diseases requires a basic understanding of developmental changes in the immune system of the rat. There are greater opportunities to investigate cell signaling and the immune system in rats due in part to the increasing availability of commercial antibodies for rats. The larger size of the rat provides more tissue/cells than the mouse for multiple measurements to explore the relationships between the immune system and physiological/pathological phenomena in the same animal.

The growth stage is a critical point in development of the immune system and is vulnerable to many environmental and genetic factors. During growth, the bone marrow (stem cell production) and thymus (T-cell maturation) are maturing, and they are also responsible for production of the cells involved in the adaptive immune response. T-cell function has been shown to be affected by such factors as malnutrition (4), disease (5, 6), and drugs (7) and these effects are amplified during growth. As T-cells mature in the thymus, their cell surface expression of CD4 and CD8 changes from double negative ($CD4^-CD8^-$) to double positive ($CD4^+CD8^+$) and eventually into single positive T-cells ($CD4^+CD8^-$ or $CD4^-CD8^+$) before being released into the periphery (8). The signaling protein $p56^{lck}$, which

is principally expressed in T-lymphocytes, is involved in the maturation of T-cells from double negative into double positive T-cells (8-11). The role of $p56^{lck}$ in thymocyte maturation has been investigated using transgenic animal models and cell culture methods (12, 13). Given the changes in $p56^{lck}$ due to dietary deficiencies (14, 15), an environmental factor, it is of interest to determine if any differences exist in the protein levels of $p56^{lck}$ along with the proportion and number of the T-cell subsets during growth in rats. Thus, the objective of the present experiment was to determine $p56^{lck}$ protein levels using Western immunoblotting and to characterize the T-cell subsets in the thymus, spleen and blood of 3, 6, and 9 week old rats using flow-cytometry. FlowCount™ Fluorospheres were used to determine absolute numbers of the T-lymphocyte subsets. Three to 9 week old rats represent the weanling phase to sexual maturity, and it takes approximately 3 weeks for a T-cell to mature upon entering the thymus (16).

6.3 Materials and Methods

6.3.1 Animals and diets

Three week old male Sprague Dawley rats (Charles River Laboratories, St. Constant, PQ) were fed a modified AIN-93G diet (14, 17) *ad libitum*. The rats were maintained in an environment of controlled temperature (21-23°C), humidity (55%) and light cycle (14 hours light/10 hours dark). Animal care was provided in accordance with a protocol approved by the Local Animal Care Committee (University of Manitoba).

6.3.2 Sample collection

At 3, 6 and 9 weeks of age, rats were killed by CO_2 asphyxiation and decapitation. Trunk blood was collected in EDTA vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ). The thymus and spleen were removed and processed immediately.

Cell preparation

Single cell suspensions of spleen and thymus in phosphate buffered saline/1% bovine fetal calf serum (Gibco, Grand Island, NY) were prepared using a loose fitting Kontes glass/glass homogenizer (Kontes, Vineland, NJ). Spleen, but not thymus, cell suspensions were gently layered over Lympholyte-Rat (Cedarlane, Hornby, ON) and centrifuged at 1500 x g for 20 minutes at room temperature to reduce presence of polymorphonuclear cells and erythrocytes. Spleen cells from the interphase layer (primarily mononuclear cells) and thymus cell suspensions were washed twice (400 x g for 10 minutes) and resuspended at a concentration of $1x10^7$/mL in phosphate buffered saline/5% fetal calf serum.

6.3.3 Outcome measurements

Determination of T-lymphocyte subpopulations

Antibodies: Monoclonal antibodies for TCR$\alpha\beta$ (PE label, R73 clone), CD4 (PC5 label, OX-35 clone), and CD8 (FITC label, G28 clone) were obtained from BD Pharminigen (Mississauga, ON). The sample combinations that were used for three-color analysis were: TCR$\alpha\beta$, CD4, CD8 or their respective isotype controls.

Cell labeling: Thymus and spleen cells (1×10^6) were incubated with the mixture of monoclonal antibodies for 40 minutes at 4°C. After addition of 3 mL cold

phosphate buffered saline/0.5% bovine serum albumin, cells were centrifuged (300 g for 6 minutes), resuspended in a fixation solution of 1% paraformaldehyde in phosphate buffered saline (pH 7.2) and kept on ice. Whole blood (100 μL) was incubated with the appropriate antibodies or isotype controls for 15 minutes, mixed with 500 μl Optilyse C (Beckman, Mississauga, ON), and subsequently prepared according to manufacturers instructions. FlowCount™ fluorospheres (100 μL, Beckman, Mississauga, ON) were added to tubes for thymus, spleen and blood. Samples were analyzed using a Beckman Coulter EPICS ALTRA (Beckman Coulter Canada, Mississauga, ON) high speed cell sorter with laser excitation tuned to 488nm (65mW). Forward versus side scatter histograms were used to gate on intact lymphoid cells. Further gating on cell surface markers is indicated in the tables and figures, and representative flow cytometry plots are shown in Figures 6.7.1-6.7.3. The fluorescence signals were separated with the standard dichronic long pass filters provided with the instrument and detected through 525 nm (FITC), 575 nm (PE) and 675 nm (PC5) bandpass filers, respectively. The data were collected in listmode format with the subsequent analyses based on 10 000 cells satisfying the light scatter gate using the EXPO32 MultiCOMP MFA software provided with the instrument. Fluorochrome-istotype matched controls were prepared to assess autofluorescence and nonspecific binding, while single color samples were employed to adjust color compensation. Absolute cell counts were calculated based on the total number of cells counted, the total number of fluorospheres

counted and the concentration of the fluorospheres. Cells counts were corrected for the weight of the thymus or spleen used to prepare the cell suspensions.

Western immunoblotting for $p56^{lck}$

Cell lysates were prepared by resuspending thymocytes and splenocytes in buffer containing protease inhibitors as previously described (14). Protein concentration was determined using the Bicinchoninic Acid Protein Assay (Sigma, St. Louis, MO). For Western blotting, cell lysates (20 μg protein per lane), molecular weight standard and positive control (Jurkat Cells Lysate, clone: Human T-cell leukemia, BD Pharmingen, Mississauga, ON) were separated by SDS-PAGE (5% stacking gel and 10% separating gel) and transferred to nitrocellulose membrane (0.2 um; BioRad, Hercules, CA) using previously published procedures (14). $P56^{lck}$ was detected using mouse anti-human lck (1:5000; clone 28, Transduction Laboratories, Lexington, KY), goat anti-mouse IgG horseradish peroxidase (1:1000) and Chemi Glow (Fisher, Whitby, ON) as the luminescent substrate. Arbitrary units for bands were determined using the FluorChem digital imaging system (Alpha Innotech Corporation, San Leandro, CA) and FluorChem software (version 2.0).

6.4 Statistical Analysis

Data were analyzed by one-way ANOVA using the general linear models procedure (SAS software release 8.2, SAS Institute, Cary, NC). When necessary, data were normalized by log transformation for statistical analyses, but non-transformed means are reported. Significant differences among means

were determined using Duncan's multiple range test. Differences were considered significant at $P<0.05$. Pearson's correlation coefficient was used to examine correlations between $p56^{lck}$ and age.

6.5 Results

Body and lymphoid organ weights. Body weight of 3 week old rats increased 60% by 6 weeks, and another 30% by 9 weeks of age (Table 6.6.1). Thymus and spleen weights increased 44% between 3 and 6 weeks, but remained similar between 6 and 9 weeks. However, thymus and spleen weights corrected to body weight decreased with age.

$P56^{lck}$. In thymus, there was a trend ($p=0.1$) for decreasing $p56^{lck}$ protein levels with increasing age (Figure 6.7.4). In spleen, there was an increase in $p56^{lck}$ protein levels between 3 and 9 weeks of age. Furthermore, $p56^{lck}$ and age were negatively correlated ($r=-0.42$, $p=0.04$) in the thymus and positively correlated ($r=0.50$, $p=0.01$) in the spleen.

$TCR\alpha\beta^+$ lymphocytes. There were no differences in the proportion of $TCR\alpha\beta^+$ cells (gated on lymphocytes) among age groups in the thymus; however, there was a lower percentage of $TCR\alpha\beta^+$ cells in the spleen and blood of 3 week old rats compared to both 6 and 9 week old rats (Table 6.6.2). Three week old rats had fewer $TCR\alpha\beta^+$ cells per g of spleen and μL of blood, and per spleen and total blood volume compared to both 6 and 9 week old rats, but no differences were detected among groups in the thymus.

Proportions of T-lymphocyte subpopulations

i) Thymus: Figures 6.7.6a and 6.7.6b represent the proportion of T-lymphocyte subsets in the thymus after gating on $TCR\alpha\beta^-$ and $TCR\alpha\beta^+$ cells, respectively. The proportion of the most immature T-lymphocyte phenotype in the thymus ($TCR\alpha\beta^-CD4^-CD8^-$) was lower in 6 week old rats compared to 9 week old rats. Both 3 and 6 week old rats had a lower proportion of $TCR\alpha\beta^-CD4^-CD8^+$ and a higher percentage of $TCR\alpha\beta^-CD4^+CD8^+$ cells (the next phenotypes in T-lymphocyte maturation) compared to 9 week old rats. The next step in maturation is $TCR\alpha\beta^+CD4^+CD8^+$ and 3 week old rats had a higher percentage of $TCR\alpha\beta^+CD4^+CD8^+$ cells, compared to 9 week old rats. The most mature T-lymphocytes found in the thymus are either $TCR\alpha\beta^+CD4^+CD8^-$ or $TCR\alpha\beta^+CD4^-CD8^+$. There were no differences among the age groups in the proportion of $TCR\alpha\beta^+CD4^+CD8^-$ cells, however, the proportion of $TCR\alpha\beta^+CD4^-CD8^+$ cells were lower in the 3 week old rats compared to 6 and 9 week old rats.

ii) Spleen: Figure 6.7.6c represents the proportion of T-lymphocyte subsets in the spleen after gating on $TCR\alpha\beta^+$ cells. The percentages of $TCR\alpha\beta^+CD4^+CD8^+$ and $TCR\alpha\beta^+CD4^+CD8^-$ cells were lower in the 3 week old rats compared to the 9 week old rats, while there were no differences among the age groups in the percentages of $TCR\alpha\beta^+CD4^-CD8^-$ or $TCR\alpha\beta^+CD4^-CD8^+$ cells.

iii) Blood: Figure 6.7.6d represents the proportion of T-lymphocyte subsets in the blood after gating on $TCR\alpha\beta^+$ cells. The percentage of $TCR\alpha\beta^+CD4^+CD8^-$ cells was lower in 3 and 6 week old rats compared to 9 week old rats, while there were

no differences among the age groups in the percentages of $TCR\alpha\beta^+CD4^-CD8^-$, $TCR\alpha\beta^+CD4^+CD8^+$ or $TCR\alpha\beta^+CD4^-CD8^+$ cells.

Absolute number of T-lymphocyte subpopulations

i) Thymus: Figures 6.7.7a and 6.7.7b show the absolute number of T-lymphocyte subpopulations in the thymus using FlowCount™ Fluorospheres and gating on $TCR\alpha\beta^-$ and $TCR\alpha\beta^+$ cells, respectively. There were fewer $TCR\alpha\beta^-CD4^-CD8^-$ cells in 3 week old rats compared to 9 week old rats, and fewer $TCR\alpha\beta^+CD4^-CD8^-$ cells in 3 week old rats compared to 9 week old rats. Three week old rats had fewer $TCR\alpha\beta^-CD4^-CD8^+$ and $TCR\alpha\beta^+CD4^-CD8^+$ cells compared to both 6 and 9 week old rats.

ii) Spleen: Figure 6.7.7c shows the absolute number of T-lymphocyte subpopulations in the spleen using FlowCount™ Fluorospheres and gating on $TCR\alpha\beta^+$ cells. Three week old rats had fewer $TCR\alpha\beta^+CD4^-CD8^-$, $TCR\alpha\beta^+CD4^+CD8^+$, $TCR\alpha\beta^+CD4^+CD8^-$, and $TCR\alpha\beta^+CD4^-CD8^+$ compared to both 6 and 9 week old rats.

iii) Blood: Figure 6.7.7d shows the absolute number of T-lymphocyte subpopulations in the total blood volume using FlowCount™ Fluorospheres and gating on $TCR\alpha\beta^+$ cells. Three week old rats had fewer $TCR\alpha\beta^+CD4^-CD8^-$, $TCR\alpha\beta^+CD4^+CD8^+$, $TCR\alpha\beta^+CD4^+CD8^-$, and $TCR\alpha\beta^+CD4^-CD8^+$ cells compared to 6 week old rats, and 6 week old rats had fewer cells of each phenotype compared to 9 week old rats.

T-Lymphocyte numbers corrected for body weight. There were no differences among the age groups in the number of thymic or splenic $TCR\alpha\beta^+$ lymphocytes

per gram of body weight, however, 3 week old rats had fewer TCR$\alpha\beta^+$ cells/g body weight in their blood compared to 6 and 9 week old rats (Figure 6.7.5).

6.6 Tables

Table 6.6.1. Body and lymphoid organ weights of 3, 6 and 9 week old rats[1].

	Age		
	3 week	6 week	9 week
Body weight, g	116 ± 2^{c}	289 ± 6^{b}	407 ± 21^{a}
Thymus weight, g	0.50 ± 0.03^{b}	0.90 ± 0.06^{a}	0.74 ± 0.06^{a}
Thymus/body weight, %	0.43 ± 0.02^{a}	0.31 ± 0.02^{b}	0.19 ± 0.01^{c}
Spleen weight, g	0.42 ± 0.02^{b}	0.74 ± 0.04^{a}	0.72 ± 0.06^{a}
Spleen/body weight, %	0.36 ± 0.02^{a}	0.25 ± 0.01^{b}	0.18 ± 0.01^{c}

[1]Values are means ± SEM for n=9 (3 week), n=8 (6 week), and n=6 (9 week). Different superscript letters indicate significant differences among means, $p < 0.05$.

Table 6.6.2. TCRαβ$^+$ expression on lymphocytes from the thymus, spleen and blood of 3, 6 and 9 week old rats[1].

	Age		
	3 weeks	6 weeks	9 weeks
Thymus			
TCRαβ$^+$			
%	71.34 ± 1.51	74.38 ± 2.11	68.23 ± 2.57
cells/g (x10^9)	105.4 ± 45.8	100.4 ± 10.2	109.9 ± 19.2
cells/thymus (x10^9)	51.96 ± 25.3	90.44 ± 10.8	76.86 ± 13.7
Spleen			
TCRαβ$^+$			
%	19.27 ± 2.45^b	33.94 ± 1.00^a	36.70 ± 2.68^a
cells/g (x10^8)	18.92 ± 5.08^b	46.49 ± 6.17^a	47.30 ± 6.94^a
cells/spleen (x10^8)	8.25 ± 2.44^b	35.14 ± 5.69^a	32.24 ± 4.27^a
Blood [2]			
TCRαβ$^+$			
%	28.86 ± 1.95^b	43.11 ± 2.36^a	48.62 ± 2.10^a
cells/µl	1975 ± 92^b	4177 ± 428^a	4283 ± 299^a
cells/total blood volume (x10^6)	12.98 ± 0.62^c	69.47 ± 7.32^b	97.99 ± 9.58^a

[1]Values are means ± SEM for n=9 (3week), n=8 (6 week), and n=6 (9 week). Different superscript letters indicate significant differences among means, P <0.05. Absolute cell numbers calculated using Flow-Count™ Fluorospheres. Percentages and absolute numbers determined after gating on total lymphocytes.

[2]Total blood volume estimated using 57.5 mL/kg (20).

6.7 Figures

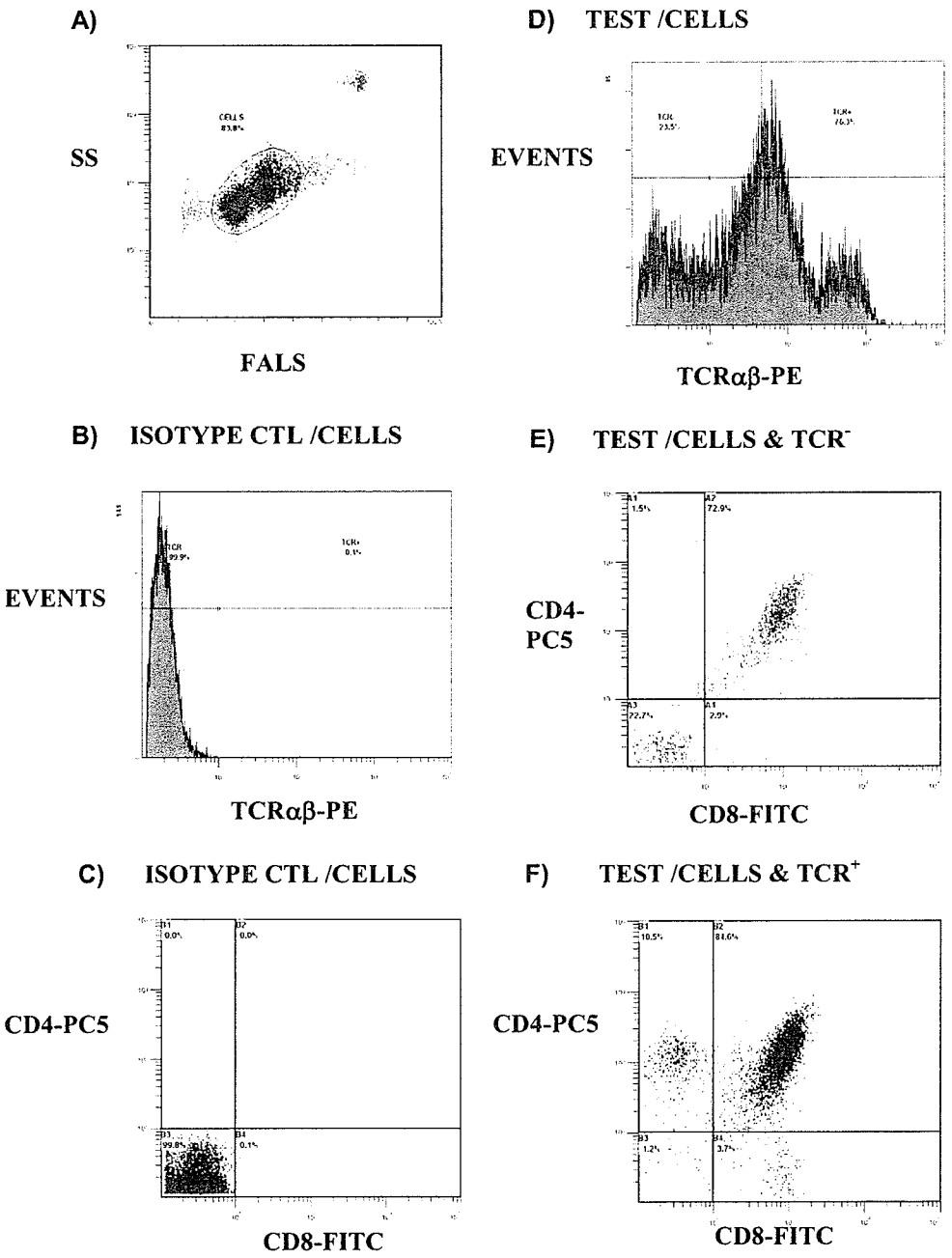


Figure 6.7.1. Representative flow cytometry plots of lymphocytes from the thymus of 6 week old rats. Definition of cell sample (lymphocytes) by light scatter (A); Isotype control sample gated on CELLS (B&C); Test sample gated on CELLS defining TCRαβ binding (D); Test sample

gated on CELLS and TCRαβ- defining $CD4^+$ and $CD8^+$ $TCR\alpha\beta^-$ lymphocytes (E); Test sample gated on CELLS and $TCR\alpha\beta^+$ defining $CD4^+$ and $CD8^+$ $TCR\alpha\beta^+$ lymphocytes (F).

Abbreviations: SS=side scatter; FALS=forward angle light scatter; CTL=control.

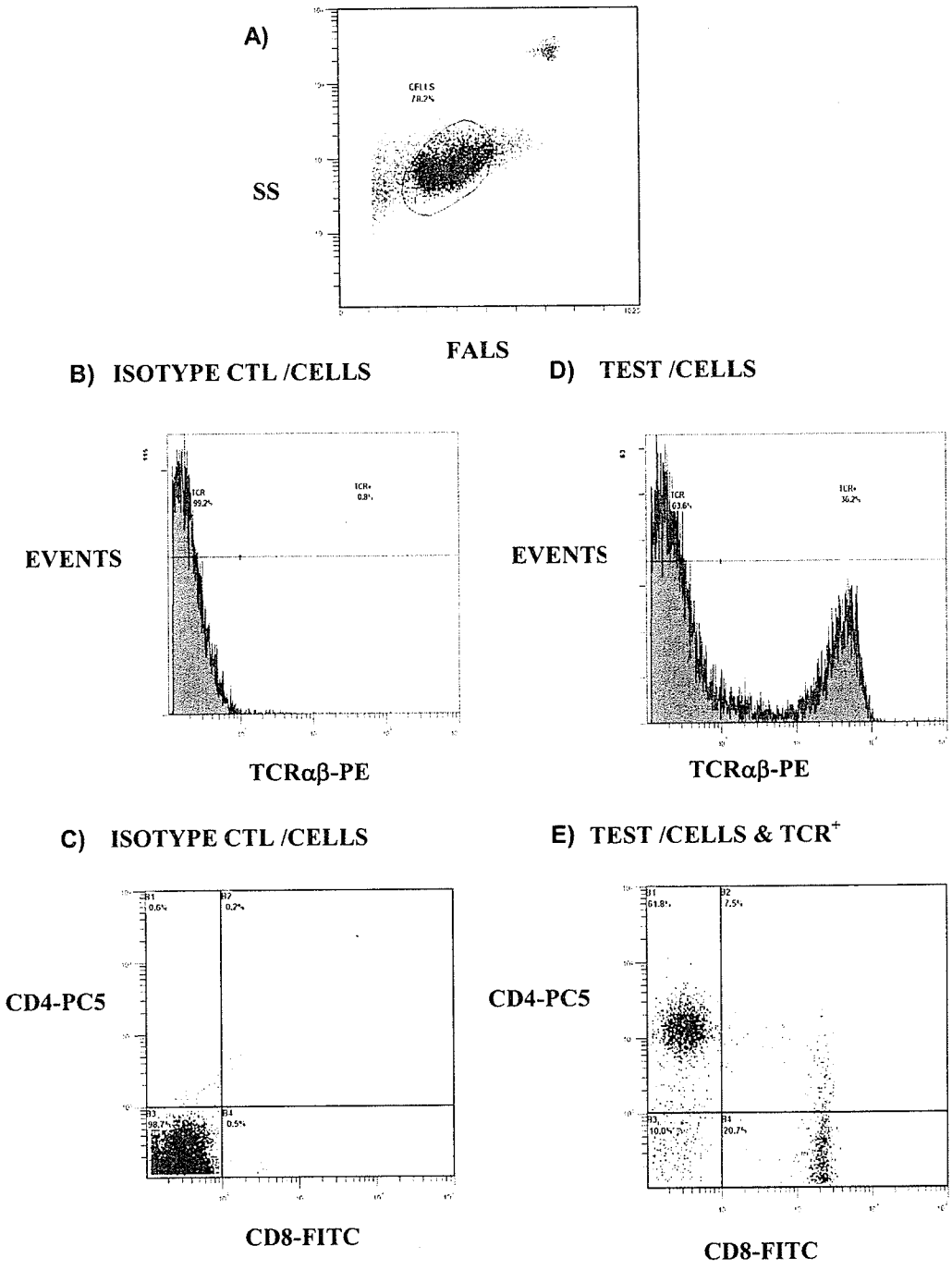


Figure 6.7.2. Representative flow cytometry plots of lymphocytes from the spleen of 6 week old rats. Definition of cell sample (lymphocytes) by light scatter (A); Isotype control sample gated on CELLS (B) and CELLS and TCRαβ$^+$ (C); Test sample gated on CELLS defining TCRαβ binding (D); Test sample gated on CELLS and TCRαβ$^+$ defining CD4$^+$ and CD8$^+$ TCRαβ$^+$ lymphocytes (E). Abbreviations: SS=side scatter; FALS=forward angle light scatter; CTL=control.

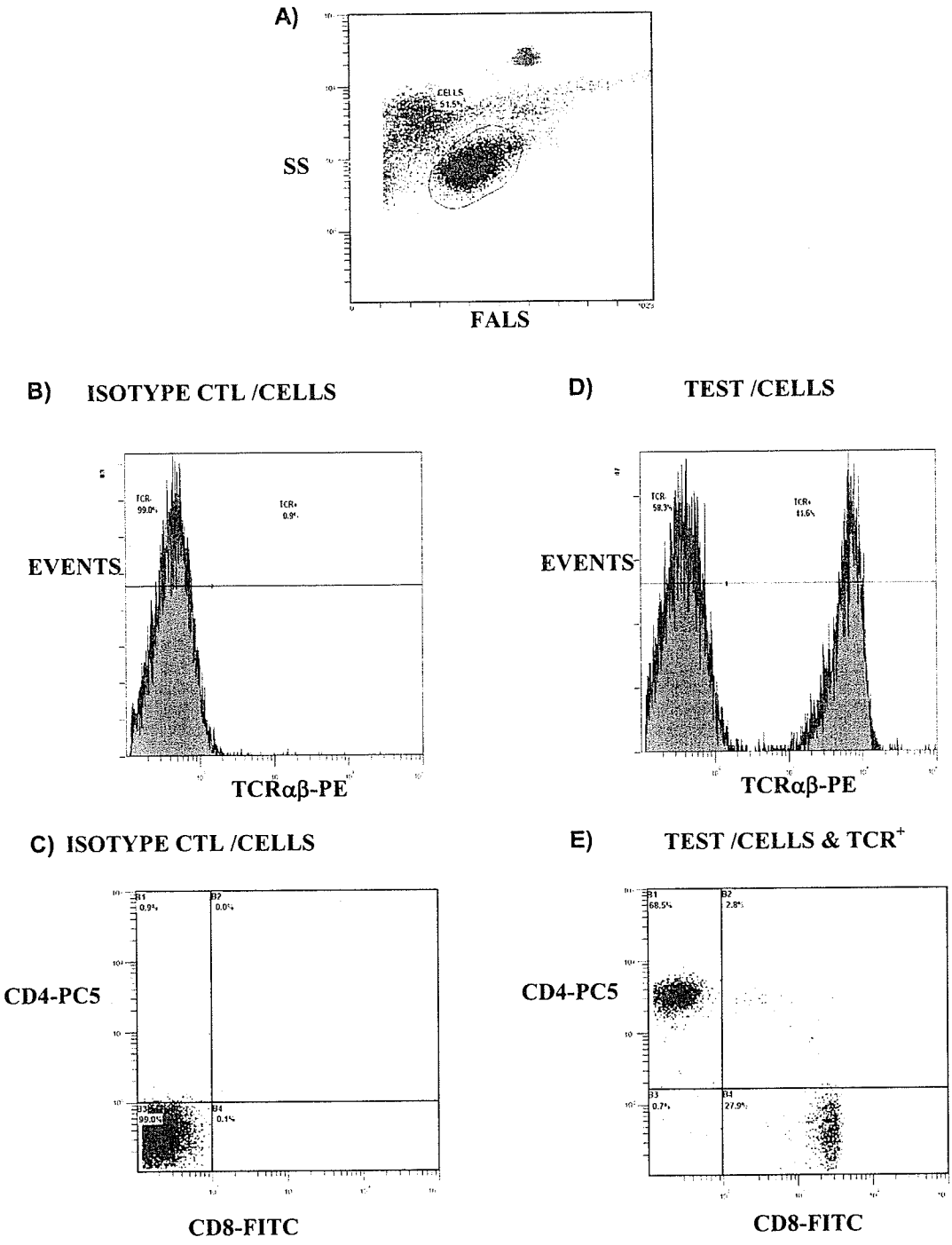


Figure 6.7.3. Representative flow cytometry plots of lymphocytes from the blood of 6 week old rats. Definition of cell sample (lymphocytes) by light scatter (A); Isotype control sample gated on CELLS (B&C); Test sample gated on CELLS defining TCRαβ binding (D); Test sample gated on CELLS AND TCRαβ$^+$ defining CD4$^+$ and CD8$^+$ TCRαβ$^+$ lymphocytes (E). Abbreviations: SS=side scatter; FALS=forward angle light scatter; CTL=control.

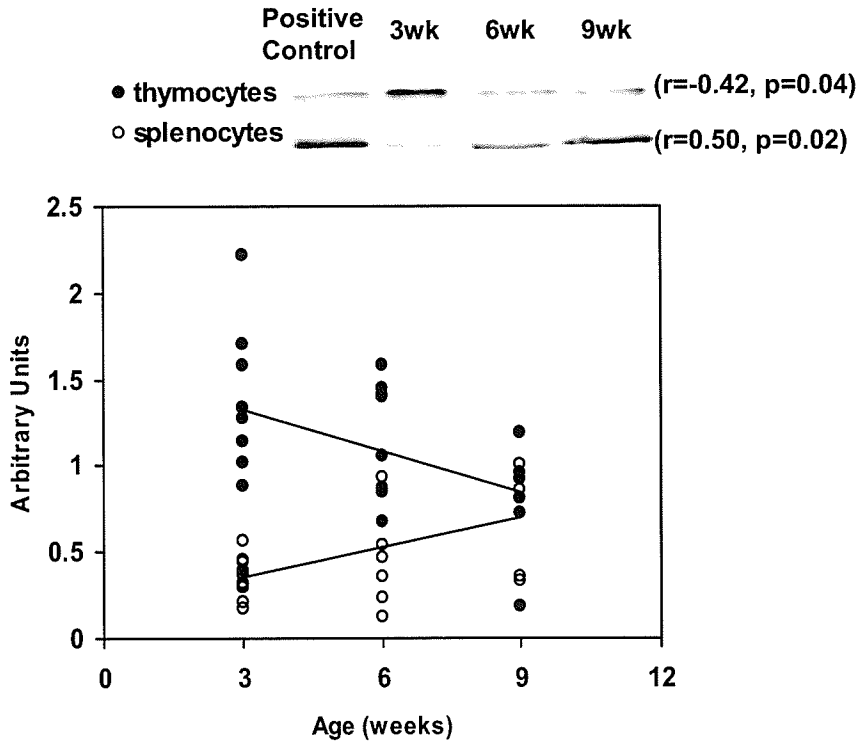


Figure 6.7.4. Representative blots and correlations of age with $p56^{lck}$ protein levels in thymocytes and splenocytes of young rats. Data points are values from individual rats (n=9, 8, 6 for 3, 6, 9 weeks, respectively).

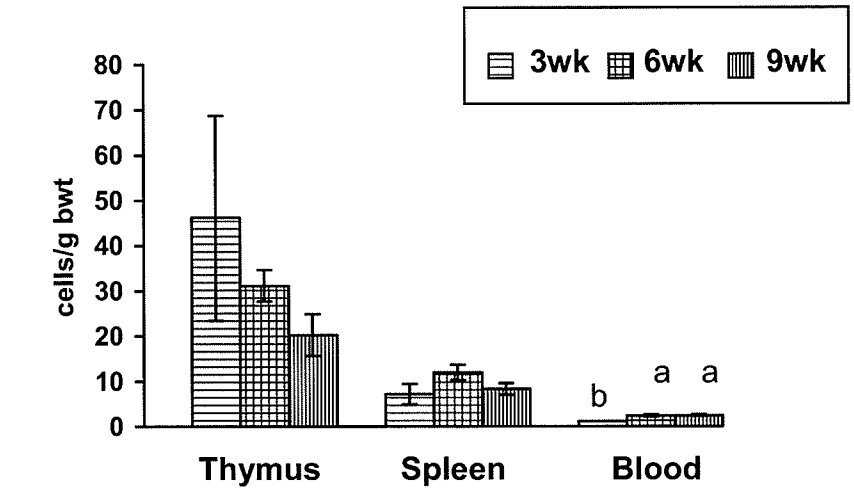


Figure 6.7.5. Absolute number of lymphocytes that positively express TCRαβ corrected for body weight. Thymus $x10^7$; Spleen $x10^6$; Blood $x10^5$. Values are means ± standard error for n=9 (3 weeks), n=8 (6 weeks) and n=6 rats (9 weeks). Different superscript letters indicates statistical differences ($p<0.05$) among age groups.

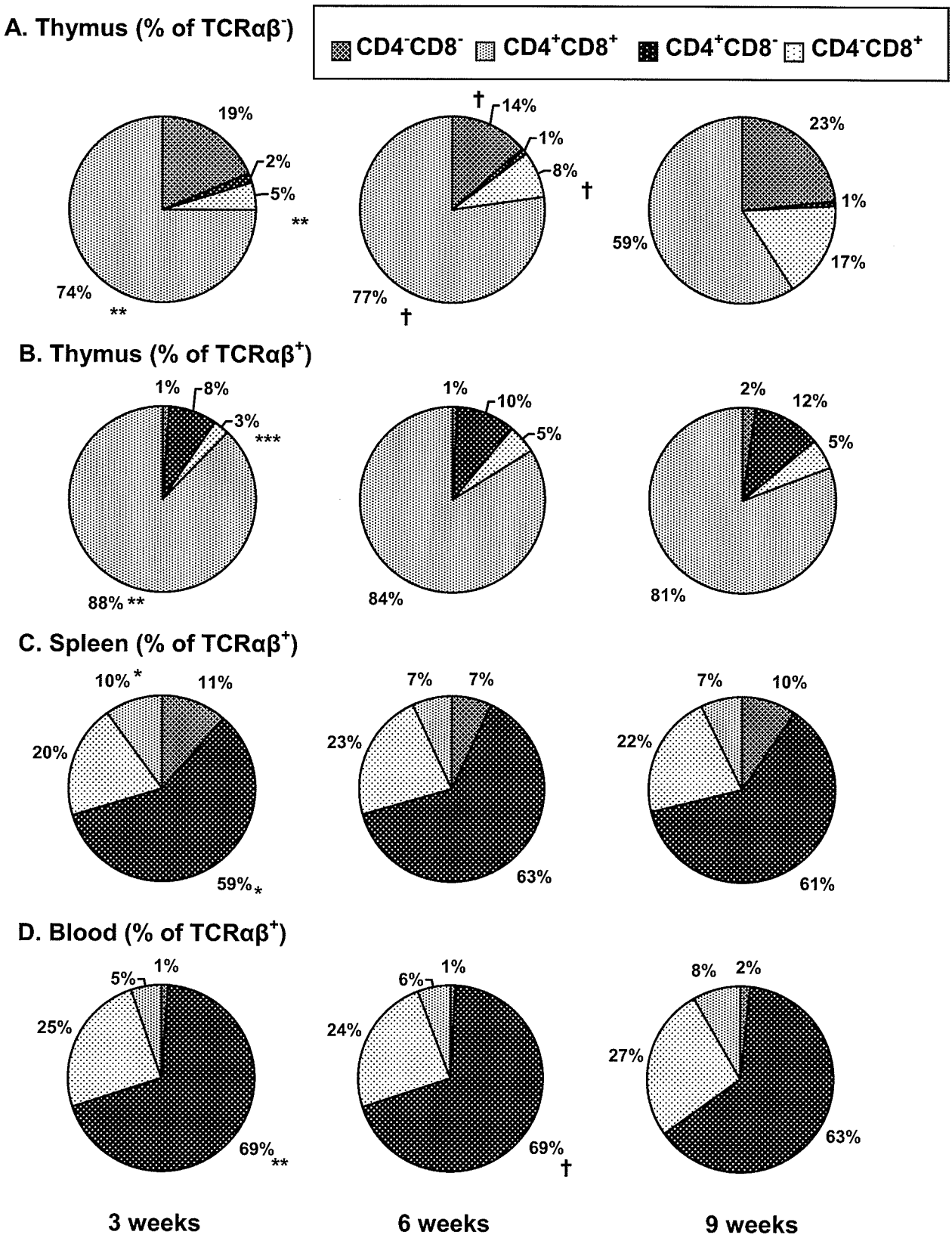

A. Thymus (% of TCRαβ-)
CD4-CD8-
CD4+CD8+
CD4+CD8-
CD4-CD8+
19%
2%
5%
**
74%
**
†
14%
1%
8%
†
77%
†
23%
1%
59%
17%
B. Thymus (% of TCRαβ+)
1%
8%
3%
***
88%**
1%
10%
5%
84%
2%
12%
5%
81%
C. Spleen (% of TCRαβ+)
10%*
11%
20%
59%*
7%
7%
23%
63%
7%
10%
22%
61%
D. Blood (% of TCRαβ+)
5%
1%
25%
69%**
6%
1%
24%
69%†
8%
2%
27%
63%
3 weeks
6 weeks
9 weeks

Figure 6.7.6. Proportions of T-lymphocyte subpopulations in the thymus (a & b), spleen (c), and blood (d) of young rats. Figure 6.6.7a was gated on TCRαβ$^-$ cells and figures 6.6.7b-d were gated on TCRαβ$^+$ cells. Values are means for n=9 (3 weeks), n=8 (6 weeks) and n=6 rats (9 weeks). Significant differences ($p<0.05$) among means are indicated by symbols: *3 week is different from 6 week; **3 week is different from 9 week; ***3 week is different from both 6 and 9 week; †6 week is different from 9 week.

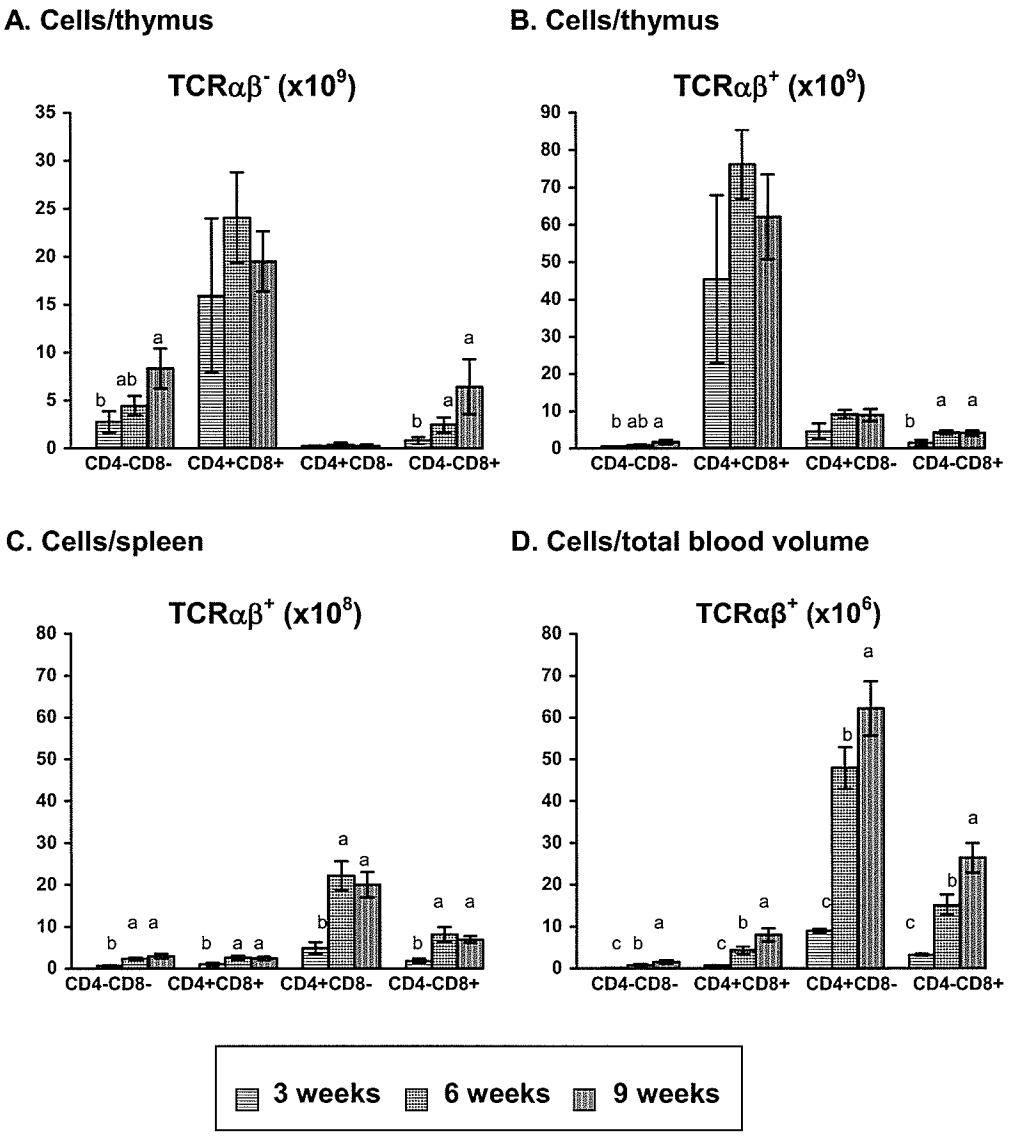


Figure 6.7.7. Absolute number of cells per thymus (a & b), spleen (c), and total blood volume (d) of young rats. Values are means ± standard error for n=9 (3 weeks), n=8 (6 weeks) and n=6 rats (9 weeks). Different superscript letters indicate statistical differences ($p<0.05$) among age groups.

6.8 Discussion

In the present study, $p56^{lck}$ protein levels decreased with age in thymic lymphocytes, but increased with age in splenic lymphocytes in 3-9 week old Sprague Dawley rats (Figure 6.7.4). $P56^{lck}$ is involved in the maturation of double negative (DN) to double positive (DP) thymocytes (8-11). There was a higher percentage of DN ($TCR\alpha\beta^{-}CD4^{-}CD8^{-}$) cells and a lower percentage of DP ($TCR\alpha\beta^{-/+}CD4^{+}CD8^{+}$) cells in the thymus of 9 week old rats compared to 3 week old rats (Figure 6.7.6) which coincides with the decrease in thymic $p56^{lck}$ levels. Previous work with cultured cells and transgenic mice has shown that strong TCR signals amplified by $p56^{lck}$ contributes to CD4 or CD8 lineage choice (12, 18). Preferential differentiation of DP to CD4 in mice and to CD8 in rats has been reported when cells from both species are stimulated *in vitro* (18). In the present study, 9 week old rats had a higher proportion of thymic single positive CD8 cells compared to 3 week old rats. This may be a reflection of higher thymocyte $p56^{lck}$ protein levels in the younger rats and the time course for T-cell maturation. Alternatively, the in vitro conditions may not reflect all the influences of the in vivo whole body environment.

There were only minor differences among the age groups in the proportion of T-cell subsets in the spleen and blood (Figure 6.7.6) indicating that the age-related changes in the thymus were not reflected in the periphery. Others have investigated T-cell maturation in the rat during the first year of life and found that in the thymus DN cells increased while DP cells decreased with age, along with no changes in T-cell subsets in the blood (19). The ages of rats in the present

study correspond to the period of rapid growth and dietary changes due to stage of life (post-lactation). The weanling rat is frequently used for investigation of dietary and environmental factors.

Three week old rats had a lower number of TCRαβ$^+$ cells per organ and per gram of spleen and μL of blood compared to 6 and 9 week old rats (Figure 6.7.7). This suggests that 3 week old rats are less equipped to respond to antigens and might leave them more susceptible to infection. However, when TCRαβ$^+$ cells were corrected for body weight there are no differences among age groups in the thymus or spleen (Figure 6.7.5) indicating that the T-cell numbers are maintained proportional to body weight in the growing rat.

6.9 Literature Cited

1. Totey SM, Kulkarni AB. Gene targeting in immunology. Indian J Exp Biol. 2000 Aug;38(8):733-45.

2. Ravirajan CT, Isenberg DA. Transgenic models of tolerance and autoimmunity: With special reference to systemic lupus erythematosus. Lupus. 2002;11(12):843-9.

3. Wong FS, Wen L. The study of HLA class II and autoimmune diabetes. Curr Mol Med. 2003 Feb;3(1):1-15.

4. Woodward B. Protein, calories, and immune defenses. Nutr Rev. 1998 Jan;56(1 Pt 2):S84-92.

5. Shou J, Ross S, Koeppen H, de Sauvage FJ, Gao WQ. Dynamics of notch expression during murine prostate development and tumorigenesis. Cancer Res. 2001 Oct 1;61(19):7291-7.

6. Kannagi M, Ohashi T, Hanabuchi S, Kato H, Koya Y, Hasegawa A, Masuda T, Yoshiki T. Immunological aspects of rat models of HTLV type 1-infected T lymphoproliferative disease. AIDS Res Hum Retroviruses. 2000 Nov 1;16(16):1737-40.

7. Colombo LL, Lopez MC, Chen GJ, Watson RR. Effect of short-term cocaine administration on the immune system of young and old C57BL/6 female mice. Immunopharmacol Immunotoxicol. 1999 Nov;21(4):755-69.

8. Zamoyska R, Basson A, Filby A, Legname G, Lovatt M, Seddon B. The influence of the src-family kinases, Ick and fyn, on T cell differentiation, survival and activation. Immunol Rev. 2003 Feb;191:107-18.

9. Abraham KM, Levin SD, Marth JD, Forbush KA, Perlmutter RM. Delayed thymocyte development induced by augmented expression of p56lck. J Exp Med. 1991 Jun 1;173(6):1421-32.

10. Molina TJ, Kishihara K, Siderovski DP, van Ewijk W, Narendran A, Timms E, Wakeham A, Paige CJ, Hartmann KU, Veillette A. Profound block in thymocyte development in mice lacking p56lck. Nature. 1992 May 14;357(6374):161-4.

11. Levin SD, Anderson SJ, Forbush KA, Perlmutter RM. A dominant-negative transgene defines a role for p56lck in thymopoiesis. EMBO J. 1993 Apr;12(4):1671-80.

12. Sohn SJ, Forbush KA, Pan XC, Perlmutter RM. Activated p56lck directs maturation of both CD4 and CD8 single-positive thymocytes. J Immunol. 2001 Feb 15;166(4):2209-17.

13. Glaichenhaus N, Shastri N, Littman DR, Turner JM. Requirement for association of p56lck with CD4 in antigen-specific signal transduction in T cells. Cell. 1991 Feb 8;64(3):511-20.

14. Lepage LM, Giesbrecht JA, Taylor CG. Expression of T-lymphocyte p56(lck), a zinc-finger signal transduction protein, is elevated by dietary zinc deficiency and diet restriction in mice. J Nutr. 1999 Mar;129(3):620-7.

15. Hosea HJ, Rector ES, Taylor CG. Zinc-deficient rats have fewer recent thymic emigrant (CD90+) T-lymphocytes in spleen and blood. J Nutr. 2003 Dec;133(12):4239-42.

16. Sharon J. Basic immunology. Baltimore: Williams and Williams; 1998.

17. Reeves PG, Nielsen FH, Fahey GC,Jr. AIN-93 purified diets for laboratory rodents: Final report of the american institute of nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr. 1993 Nov;123(11):1939-51.

18. Mitnacht R, Bischof A, Torres-Nagel N, Hunig T. Opposite CD4/CD8 lineage decisions of CD4+8+ mouse and rat thymocytes to equivalent triggering signals: Correlation with thymic expression of a truncated CD8 alpha chain in mice but not rats. J Immunol. 1998 Jan 15;160(2):700-7.

19. Capri M, Quaglino D, Verzella G, Monti D, Bonafe M, Cossarizza A, Troiano L, Zecca L, Pasquali-Ronchetti I, Franceschi C. A cytofluorimetric study of T-lymphocyte subsets in rat lymphoid tissues (thymus, lymph nodes) and peripheral blood: A continuous remodelling during the first year of life. Exp Gerontol. 2000 Aug;35(5):613-25.

20. Olfer ED, Cross BM, McWilliam AA. Guide to the care and use of experimental animals. Vol. 1. Ottawa: Canadian Council on Animal Care; 1993.

7. THE LOWER PROPORTION OF SPLENIC CD90$^+$ T-CELLS IN ZINC-DEFICIENT RATS IS DUE TO A SMALLER PROPORTION OF LATE THYMIC EMIGRANTS^

Heather J. Hosea[1], Edward S. Rector[2], and Carla G. Taylor[1]

Departments of Human Nutritional Sciences[1] and Immunology[2]

^This research was presented in part at the CSCN 5th Annual Scientific Meeting, Healthy Aging, Inflammation in acute & Chronic Diseases, in Edmonton, Alberta, May 25-26, 2006.

Hosea H, Rector E , Taylor C. The lower proportion of CD90$^+$ T-cells in zinc-deficient rats is due to a smaller proportion of late thymic emigrants. Proceedings of the CSNS 5th Annual Scientific Meeting. 2006; Abstract 25.

7.1 Abstract

Zinc-deficient rats have a lower proportion of splenic $CD90^+$ T-cells which could be due to fewer new T-cells exiting the thymus, defective post-thymic maturation or increased cell death. Post-thymic maturation of splenic lymphocytes and their viability were determined by flow cytometry in weanling rats assigned to: Zinc-deficient (ZD, <1 mg zinc/kg, *ad libitum*), diet-restricted (DR, 30 mg zinc/kg, limited to the amount of feed as consumed by ZD), marginally zinc-deficient (MZD, 10 mg zinc/kg, *ad libitum*) or control (CTL, 30 mg zinc/kg, *ad libitum*) groups for 3 weeks. Although the overall proportion of cells in the lymphocyte and non-viable gates were not affected by diet, ZD rats had a 242% higher proportion of granulocytes compared to the other groups. ZD rats had a 29% lower percentage of splenic $CD90^+$ T-cells and both ZD and DR had a 30% lower proportion of splenic $CD90^+$ B-cells compared to CTL. When the splenic $CD90^+$ T-cells were characterized further, there was no difference among the groups in the first two stages of post-thymic development; however, ZD, DR and MZD had a 42% lower proportion of late thymic emigrants ($TCR\alpha\beta^+CD90^+CD45RC^+RT6.1^+$) compared to CTL. There was no difference among groups in the proportion of splenic $CD90^+$ T-cells in the non-viable region, however, ZD did have a higher proportion of $CD90^+$ B-cells in the non-viable region compared to MZD and CTL, suggesting that this phenotype was more susceptible to cell death during deficiency. The lower proportion of splenic $CD90^+$ T-cells in ZD rats does not appear to be due to a defect in thymic

production or increased cell death in the spleen. Future studies should determine if late thymic emigrants have homed to other peripheral organs.

7.2 Introduction

The immune system protects us from infection and disease (1). A diverse T-cell receptor repertoire is essential for the recognition of the billions of potential foreign invaders (2). In order to ensure a diverse repertoire, the thymus continuously produces and releases new cells that enter the periphery (3). Apoptosis also plays an important role in the diversification of the T-cell pool during positive and negative selection of T-cells in the thymus and removal of peripheral T-cells after activation or due to anergy (4). Dietary zinc-deficiency adversely affects the immune system leaving the individual susceptible to disease (5). Decreased production of new T-cells by the thymus or increased removal of peripheral T-cells by apoptosis could play a role in the immunodeficiency of dietary zinc deficiency.

Zinc-deficient rats have a lower proportion of $CD90^+$ T-cells in their blood and spleen, but not thymus, compared to diet-restricted and control rats (6). CD90 first appears on cortical thymocytes and is found on newly released peripheral T-cells for approximately 3 days in rats (7). Initially, both RT6 and CD45RC are expressed as CD90 disappears (3-11 days post-thymus) and 76 days after T-cells have been released from the rat thymus they express either CD45RC or RT6 (8). RT6.1 is exclusive to peripheral T-cells and expressed by ~70% of peripheral T-cells (9). The function of $RT6^+$ T-cells is still under investigation, but they have been described as immunoregulatory cells that play a role in graft-vs-host reactions (10), and prevention of autoimmunity (11). CD45RC is found on pre B-cells, B-cells, CD8+ T-cells and $CD4^+$ T-cells, but on

very few thymocytes (12). Both T- and B- cell receptor signal transduction involves CD45 (13). Expression of CD45RC on T-cells seems to increase activation (production of IL-2) and shift cytokine production in favor of Th1 type cytokines (IL-2 and IFNγ) (14). CD45RC positive cells have been shown to provoke graft-vs-host reactions and organ-specific autoimmune diseases (15, 16). In the rat, CD45RC can also be used to identify naïve and revertant memory T-cells from recently activated T-cells (15, 17). Further characterization of the maturation of $CD90^+$ T-cells into $CD90^-$ T-cells using markers such as RT6.1 and CD45RC has not been investigated in a model of dietary zinc deficiency. It is possible that the fewer $CD90^+$ T-cells in ZD could be due to fewer new T-cells exiting the thymus or due to defective post-thymic maturation.

Fraker (18) has proposed that the mechanism that best explains the lymphopenia and immunodeficiency in zinc deficiency is increased apoptosis rates in the immature lymphocytes of the thymus and bone marrow. Zinc deficiency studies in the adult mouse have indicated that pre-T-cells ($CD4^+CD8^+$ thymocytes) show enhanced rates of apoptosis compared to control mice (19), and there is a lower proportion of pre-B-cells ($CD45R^+CD43^-IgM^-$) in the bone marrow of zinc-deficient mice compared to control mice (20).

In the rat, the apoptotic susceptibility of peripheral lymphocytes has not been determined in zinc-deficiency. It has been reported however that diabetes-prone BioBreeding rats have a higher proportion of intrahepatic T-cells undergoing apoptosis, most of which are recent thymic emigrants ($CD90^+RT6^-$ T-cells), compared diabetes-resistant rats (21). We hypothesize that zinc

deficiency alters the proportion of splenic T-cell phenotypes (either by altered post-thymic maturation or increased cell death), leaving fewer new cells in the spleen (peripheral lymphoid organ) and thereby limiting the lymphocyte repertoire.

The objective of the present study was to determine the proportion of lymphocytes that express different combinations of TCRαβ, CD90, RT6.1 and CD45RC to further characterize the stage of maturity of splenic T- and B-cells in both marginal and severe zinc deficiency using the growing rat as a model. A secondary objective was to determine whether differences in the proportion of splenic lymphocyte subsets could be explained by increased/decreased non-viability. Fraker and colleagues (22) have shown that cell viability can be identified using flow cytometry and forward versus side scatter to separate the smaller non-viable cells from the slightly larger viable cells. The authors acknowledge that this method is not the most sensitive and does not discriminate apoptotic cells from the necrotic cells inevitably produced during preparation of cells for flow cytometric analysis; however, it does provide a crude estimation of the non-viable (apoptotic plus necrotic) lymphocyte phenotypes in the given sample population. In the present experiment, we examined the lymphocyte phenotypes of cells that appeared in the non-viable region to determine if one particular phenotype was more or less prominent. We included both a zinc-deficient group [<1 mg zinc/kg; characterized by anorexia and growth arrest (23)] as well as a marginally zinc-deficient group [10 mg zinc/kg; no expected effect on

growth], as the effects of marginal zinc deficiency on immunity during growth may be relevant to human nutrition.

7.3 Materials and Methods

7.3.1 Animals and diets

Twenty one day old Sprague Dawley rats (Charles River Laboratories, St. Constant, PQ) were acclimatized for 5 days and randomly assigned to one of four dietary treatment groups for three weeks: zinc-deficient (ZD; <1 mg zinc/kg), marginally zinc-deficient (MZD; 10 mg zinc/kg), nutritionally complete control diet (CTL; 30 mg zinc/kg), and diet-restricted (DR; fed the control diet, but only the amount of feed as consumed on the previous day by the individual zinc-deficient rat that was paired to the DR rat). The diet-restricted group controls for the effects of undernutrition commonly seen in zinc deficiency. Body weight was measured weekly and feed intake was measured daily.

The experimental diets (based on the AIN-93G formulation) containing egg white, additional biotin (2 mg/kg diet), and potassium phosphate (5.4 g/kg diet for the growth formulation) were described previously (24). The diets were fed *ad libitum*, except for the diet-restricted group, and distilled water was provided. Zinc content of the diets was verified by atomic absorption analysis. To avoid zinc recycling and contamination, the rats were housed in stainless steel hanging cages with mesh bottoms so urine and feces could drop to the shavings below. In addition, the zinc-deficient rats were placed on the upper rows of the cage rack. The rats were maintained in an environment of controlled

temperature (21-23°C), humidity (55%) and light cycle (14 hours light/10 hours dark). Animal care was provided in accordance with a protocol approved by the University of Manitoba Protocol Management and Review Committee.

7.3.2 Sample collection

At the end of the feeding trial, the rats were euthanized by CO_2 asphyxiation and decapitation. Trunk blood was collected and centrifuged (Beckman TJ-6R Tabletop centrifuge) to obtain serum. Serum was stored at -80°C until analysis. The spleen was removed aseptically, weighed and then processed immediately. Femurs were removed, cleaned of soft tissue and stored at -20°C until analysis.

Cell Preparation

Single cell suspensions from the spleens were prepared by pressing tissues through nylon screens into Hank's buffered saline containing 10 mM HEPES, 4% dextran charcoal absorbed fetal bovine serum (Hyclone, Logan, UT), and 1% antibiotic/antimycotic at pH 7.4. Dextran charcoal absorbed fetal bovine serum was used to limit the amount of external steroids that the splenocytes are exposed to during cell preparation. Red blood cells were lysed using ammonium chloride lysis buffer (155 mM NH_4Cl, 0.1 mM EDTA, 10 mM $KHCO_3$, pH 7.2). Cell concentration and viability were determined using trypan blue dye exclusion on an AO Bright-Line Hemacytometer (American Optical Corporation, Buffalo, NY).

7.3.3 Outcome measurements

Zinc analysis

After obtaining wet and dry weights, femurs and diet samples were wet-ashed using trace element grade nitric acid. After appropriate dilution of digests or serum, zinc concentration was determined by atomic absorption spectrometry (Varian Spectra AA, Varian Australia, Mulgrave, VI). Bovine liver standard reference material 1577b (US Department of Commerce, National Institute of Standards and Technology, Gaithersburg, MD) was used as quality control.

Determination of splenic T-lymphocyte subpopulations

Antibodies: Anti-rat monoclonal antibodies against TCRαβ (PE label, clone R73, isotype mouse $IgG_{1,k}$), CD90 (PerCP label, Thy1.1, clone OX-7, isotype, mouse $IgG_{1,k}$), RT6.1 (purified, clone P4/16, isotype, rat $IgG_{2b,k}$), $IgG_{2b,k}$ (biotin label, RG7/11.1 clone, isotype mouse $IgG_{2b,k}$) and CD45RC (FITC label, clone OX-22, isotype mouse $IgG_{1,k}$) were obtained from BD Pharmingen (Mississauga, ON). Streptavidin-PE-Cy7 conjugate was used to fluorescently label the biotinylated antibody.

Cell labeling and flow cytometry: Cells were washed and placed in label buffer (phosphate buffered saline containing 23 mM sodium azide and 2% FBS, pH 7.4) at 4°C at a concentration of 1×10^6 cells/mL. Purified antibodies (RT6.1) were added first, incubated for 30 minutes at 4°C, followed by a wash step. The biotinylated antibody was added next, incubated as previously described followed by a wash step. Finally, the directly conjugated antibodies and streptavidin-PE-Cy7 were added, incubated as previously described, followed by a wash step.

Cells were resuspended in 1% paraformaldehyde and stored overnight at 4°C before analysis.

Flow cytometric analysis was performed on a Beckman Coulter EPICS ALTRA (Beckman Coulter, Mississauga, ON) high-speed cell sorter with laser excitation tuned to 488 nm (65 mW). Forward versus side scatter histograms were used to identify intact lymphoid cells, non-viable cells, or granulocytes. A representative flow cytometry plot, depicting the gating strategy, is shown in Figure 7.7.1. The fluorescence signals were separated with the standard dichotic long pass filters provided with the instrument and detected through 525 nm (FITC), 575 nm (PE), 675 nm (PerCP) and 735 nm (PE-Cy7) bandpass filters. The data were collected in listmode format with the subsequent analyses based on 10 000 cells satisfying the viable light scatter gate for TCR$\alpha\beta^{+}$ using the EXPO32 MultiCOMP MFA software provided with the instrument. Flurochrome-isotype matched controls were prepared to assess autofluorescence and nonspecific binding, while single color samples were employed to adjust color compensation.

7.4 Statistical Analyses

Data were analyzed by one-way ANOVA using the general linear models procedure (SAS software release 8.2, SAS Institute, Cary NC). Significant differences among means were determined using Duncan's multiple range test. Differences were considered significant at $p \leq 0.05$.

7.5 Results

Body weight, feed intake, lymphoid organ weight and zinc status. The body weights among the dietary treatment groups were not different at the start of the trial. After the 3 week feeding trial, ZD had a 48% lower body weight and feed intake compared to MZD and CTL (Table 7.6.1). Despite consuming the same amount of feed, ZD weighed 15% less than DR. There were no differences among the dietary treatment groups for lymphoid organ weight when corrected for body weight. ZD had 65-74% lower serum and femur zinc concentrations compared to MZD, MZD had 23-24% lower serum and femur zinc concentrations compared to DR, and DR had 15% lower serum and femur zinc concentrations compared to CTL.

Size and granularity of splenocytes. Lymphocytes are of a certain size and granularity and can be readily identified in flow cytometry using forward (size) and side (granularity) scatter. Representative light scatter plots from each of the dietary treatment groups, including the means and standard error for each of the gates are shown in Figure 7.7.2. There was no difference in the proportion of splenocytes that appeared in the lymphocyte gate among treatment groups.

One of the early indicators of apoptosis is cell shrinkage which can be identified by a decrease in forward light scatter. Necrotic cells and debris might also appear in this region. There was also no difference among the dietary treatment groups in the proportion of cells that appeared in the non-viable region.

There was a difference, however, in the proportion of granulocytes, which appear slightly above the lymphocyte gate (increased side scatter) indicating

increased granularity compared to the lymphocytes. No granulocyte markers were included in the flow cytometric analysis and the cells in the granulocyte gate did not express any of the markers used in the present study (TCRαβ, CD90, CD45RC or RT6.1). The ZD group had 242% more cells in the granulocyte gate than DR, MZD and CTL.

Proportions of splenic T- and B- lymphocytes. ZD had 39% more splenic T-cells (identified as $TCR\alpha\beta^{+}$) compared to MZD and CTL in the lymphocyte gate (Figure 7.7.3a). There were no differences among dietary treatment groups in the proportion of splenic B-cells (identified as $TCR\alpha\beta^{-}$ $CD45RC^{+}$) in the lymphocyte gate (Figure 7.7.3b). In the non-viable gate there were no differences among dietary treatment groups in the proportion of splenic T- and B-cells (Figure 7.7.3c-d). To determine whether there were differences among dietary treatment groups in the susceptibility of splenic T- and B-cells to cell death, we calculated the ratio of viable:non-viable to take into account differences in the proportion of populations in the viable region. There were no differences among dietary treatment groups in the viable:non-viable ratio for splenic T- or B-cells (data not shown).

Proportions of splenic $CD90^{+}$ T- and B- lymphocytes. ZD had a 29% lower proportion of immature splenic T-cells (identified as $TCR\alpha\beta^{+}CD90^{+}$) in the lymphocyte gate compared to CTL, but ZD was not significantly different from DR and MZD (Figure 7.7.4a). Both ZD and DR had an approximately 30% lower proportion of immature B-cells (identified as $TCR\alpha\beta^{-}CD45RC^{+}CD90^{+}$) in the lymphocyte gate compared to MZD and CTL (Figure 7.7.4b). There was no

significant difference among dietary treatment groups in the proportion of splenic $CD90^+$ T-cells in the non-viable gate (Figure 7.7.4c). ZD had a 61-73% higher proportion of immature B-cells in the non-viable gate compared to CTL and MZD, but was not different from DR. DR did not have a different proportion of immature B-cells in the non-viable region compared to CTL, but did have a 41% higher proportion of immature B-cells in the non-viable region compared to MZD. The ratio of viable:non-viable $CD90^+$ T-cells was not different among the dietary treatment groups (data not shown), however, ZD and DR had an approximately 54% lower ratio of viable:non-viable immature B-cells (16.6 ± 2.5 and 22.3 ± 3.6, respectively) compared to MZD and CTL (40.7 ± 4.91 and 44.4 ± 9.3, respectively).

Stages of splenic T- lymphocyte maturity. There were no differences among the dietary treatment groups in the proportion of the most recent thymic emigrants ($TCR\alpha\beta^+CD90^+CD45RC^-RT6.1^-$) or the first stage of late thymic emigrants($TCR\alpha\beta^+CD90^+CD45RC^-RT6.1^+$) (Table 7.6.2). However, at the next stage of maturation, ZD, DR and MZD had 42% fewer late thymic emigrants ($TCR\alpha\beta^+CD90^+CD45RC^+RT6.1^+$) compared to CTL. There was no difference in the proportion of late thymic emigrants in the non-viable region and the viable:non-viable ratio among dietary treatment groups (data not shown). There were no differences among ZD, DR and CTL in the proportion of early mature peripheral T-cells ($TCR\alpha\beta^+CD90^-CD45RC^+RT6.1^+$), but MZD had 40% fewer of these cells compared to all the other dietary treatment groups. Once again, there was no difference in the proportion of early mature peripheral T-cells in the non-

viable region and the viable:non-viable ratio among dietary treatment groups (data not shown). There were no differences among the dietary treatment groups at the intermediate and end stages of T-cell maturation.

7.6 Tables

Table 7.6.1. Body weight, feed intake, lymphoid organ weights, and zinc status of zinc-deficient, marginally zinc-deficient, diet-restricted, and control rats[1].

	Dietary Group[2]			
	ZD	DR	MZD	CTL
Initial body weight (g)	105 ± 3.0	110 ± 4.1	107 ± 3.2	105 ± 2.9
Final body weight (g)	142 ± 3.5^{c}	168 ± 3.0^{b}	274 ± 4.5^{a}	280 ± 4.5^{a}
Feed intake	220 ± 7.6^{b}	227 ± 7.7^{b}	414 ± 10.1^{a}	422 ± 21.3^{a}
Thymus:bwt [(3)]	3.0 ± 0.1	3.3 ± 0.2	3.3 ± 0.1	2.8 ± 0.3
Spleen:bwt [(3)]	2.7 ± 0.1	2.4 ± 0.1	2.8 ± 0.1	2.6 ± 0.1
Serum zinc (µmol/L)	5.1 ± 0.3^{d}	25.2 ± 0.7^{b}	19.4 ± 1.0^{c}	29.8 ± 1.5^{a}
Femur zinc (µmol/g dry bone)	1.09 ± 0.05^{d}	4.14 ± 0.12^{b}	3.13 ± 0.05^{c}	4.85 ± 0.16^{a}

[1]Values are means ± SEM, n=6. Different superscript letters indicate significant differences among means, $p \leq 0.05$.

[2]ZD=zinc-deficient group; DR=diet-restricted group; MZD=marginally zinc-deficient group; CTL=control group; bwt=body weight.

[3]lymphoid organ:bwt= lymphoid organ weight (mg)/body weight (g).

Table 7.6.2. Flow cytometric analysis of splenic T-lymphocyte subpopulations of zinc-deficient, marginally zinc-deficient, diet-restricted, and control rats based on Kaminga and colleague's (1997) theory of post-thymic T-lymphocyte development in the rat[1].

	PERIPHERY						
	Immature Peripheral T-cells			Mature Peripheral T-cells			
THYMUS →	*Recent Thymic Emigrants* →	*Late Thymic Emigrants* →		*Early* →	*Inter-mediate* →	*Late* →	
CD90$^+$ CD45RC$^-$ RT6.1$^-$	CD90$^+$ CD45RC$^-$ RT6.1$^-$	CD90$^+$ CD45RC$^-$ RT6.1$^+$	CD90$^+$ CD45RC$^+$ RT6.1$^+$	CD90$^-$ CD45RC$^+$ RT6.1$^+$	CD90$^-$ CD45RC$^-$ RT6.1$^-$	CD90$^-$ CD45RC- RT6.1+ **or**	CD90$^-$ CD45RC$^+$ RT6.1$^-$
	0-1 day	1-3 days		3-11 days	11-76 days	76+ days	
ZD	7.3 ± 0.7	3.0 ± 0.4	3.9 ± 0.7^b	19.3 ± 1.4^a	24.2 ± 2.6	38.0 ± 3.1	3.4 ± 0.7
DR	8.6 ± 0.7	3.3 ± 0.5	3.8 ± 0.3^b	17.8 ± 1.9^a	18.3 ± 2.7$^{(3)}$	44.1 ± 1.6$^{(4)}$	3.3 ± 0.6
MZD	8.6 ± 0.6	3.6 ± 0.4	3.4 ± 0.4^b	11.3 ± 1.0^b	22.9 ± 2.4	45.1 ± 2.9	3.5 ± 0.7
CTL	6.4 ± 1.0	3.7 ± 0.4	6.4 ± 0.8$^{a(2)}$	19.8 ± 1.9^a	17.0 ± 2.4	38.9 ± 2.9	4.2 ± 0.6

[1]Values are means ± SEM, n=6 (except n=5 as indicated in footnotes 2-4), and gated on lymphocytes and TCRαβ$^+$. Different superscript letters within a column indicate significant differences among means, p ≤0.05. Abbreviations: ZD=zinc-deficient group; DR=diet-restricted group; MZD=marginally zinc-deficient group; CTL=control group.

[2-4]Data point removed from analysis because it was greater than 3 standard deviations from the mean (value=13.4, 49.4, 10.7, respectively).

7.7 Figures

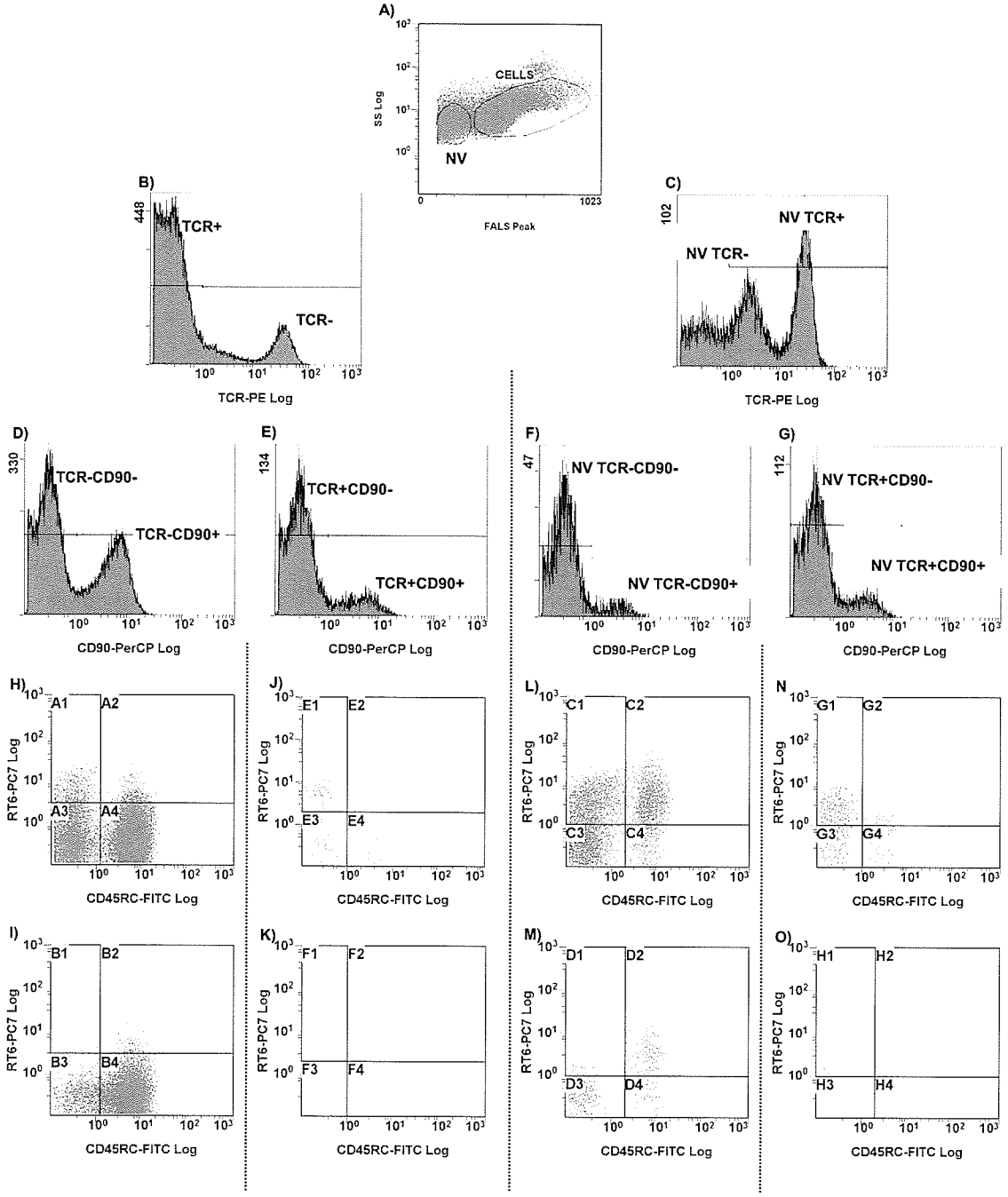


Figure 7.7.1. Representative flow cytometry plots. Definition of viable lymphocytes (CELLS) and non-viable lymphocytes (NV)[A]; Definition of TCRαβ binding after gating on CELLS [B] or NV [C]; Definition of CD90 binding after gating on CELLS that are TCRαβ$^-$ [D], CELLS that are TCRαβ$^+$ [E], NV that are TCRαβ$^-$ [F], or NV that are TCRαβ$^+$ [G]; RT6.1 and CD45RC binding on CELLS that are TCRαβ$^-$CD90$^-$ [H], CELLS that are TCRαβ$^-$CD90$^+$ [I], CELLS that are

TCRαβ^{+}CD90^{-} [J], CELLS that are TCRαβ^{+}CD90^{+} [K], NV that are TCRαβ^{-}CD90^{-} [L], or NV that are TCRαβ^{-}CD90^{+} [M], NV that are TCRαβ^{+}CD90^{-} [N], and NV that are TCRαβ^{+}CD90^{+} [O].

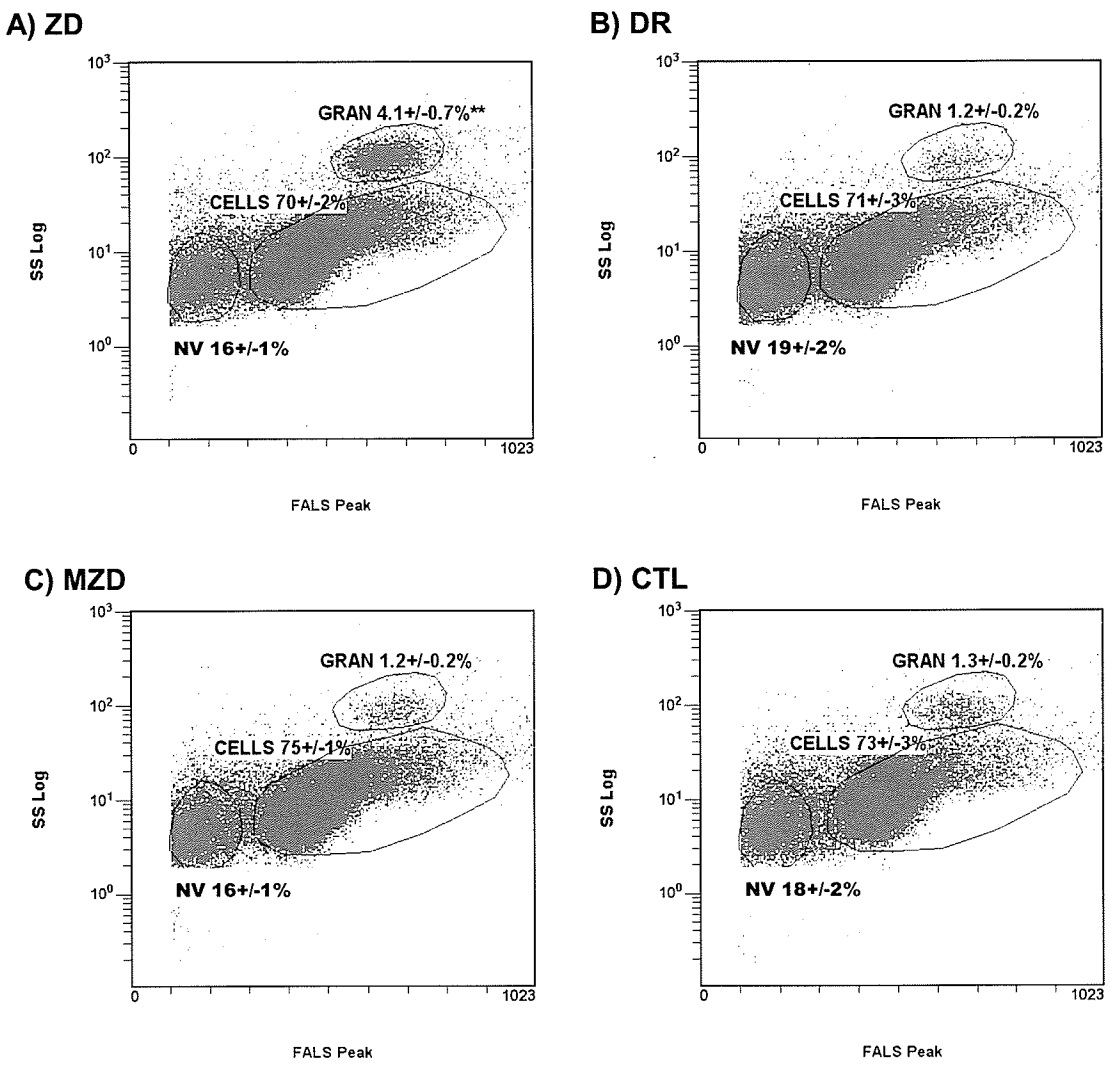


Figure 7.7.2. Forward versus side scatter histograms of splenocytes from zinc-deficient, marginally zinc-deficient, diet-restricted and control rats. Representative histograms showing regions for viable lymphocytes (CELLS), non-viable cells (NV), and granulocytes (GRAN). Values are means ± SEM for n=6 rats. ** indicates significantly different from other dietary treatment groups ($p \leq 0.05$).

Abbreviations: ZD=zinc-deficient group; DR=diet-restricted group; MZD=marginally zinc-deficient group; CTL=control group.

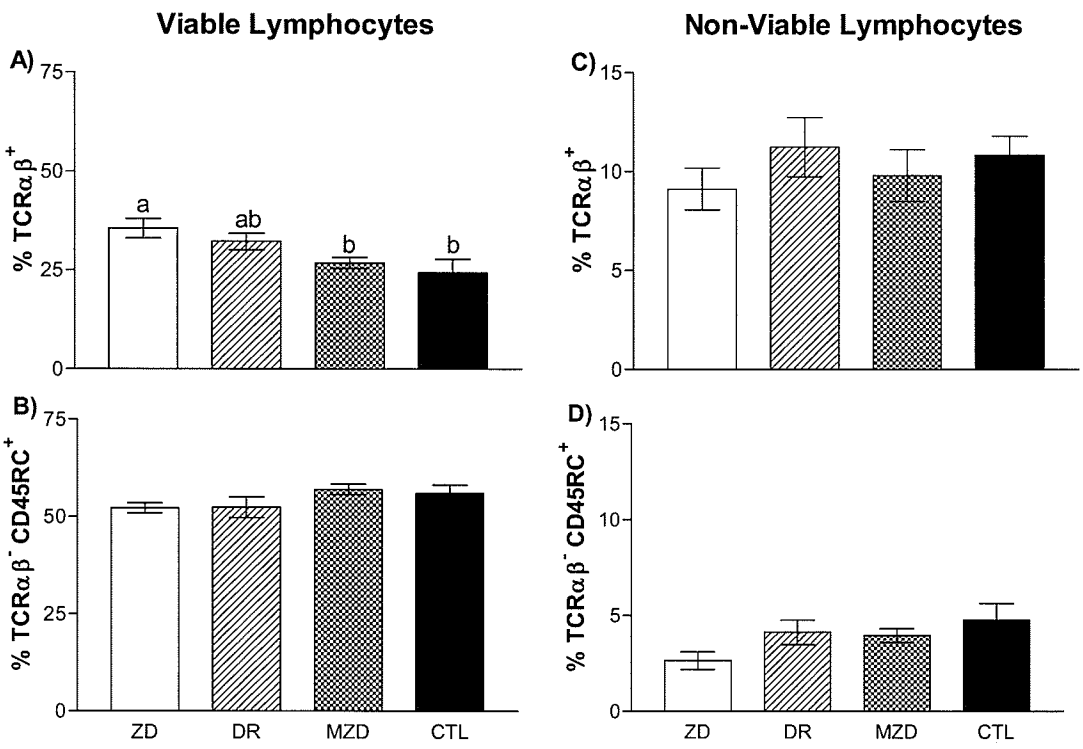


Figure 7.7.3: Proportion of splenic T- and B- lymphocytes (viable and non-viable) from zinc-deficient, marginally zinc-deficient, diet-restricted and control rats. Proportion of TCRαβ$^+$ cells (T-cells) in lymphocyte gate [A]; Proportion of TCRαβ$^-$ CD45RC$^+$ cells (B-cells) in lymphocyte gate [B]; Proportion of TCRαβ$^+$ cells in non-viable gate [C]; Proportion of TCRαβ$^-$ CD45RC$^+$ cells in non-viable gate [D]. Values are means ± SEM for n=6 rats. Different letters indicate significant differences among means ($p \leq 0.05$, except [D] $p=0.0547$).

Abbreviations: ZD=zinc-deficient group; DR=diet-restricted group; MZD=marginally zinc-deficient group; CTL=control group.

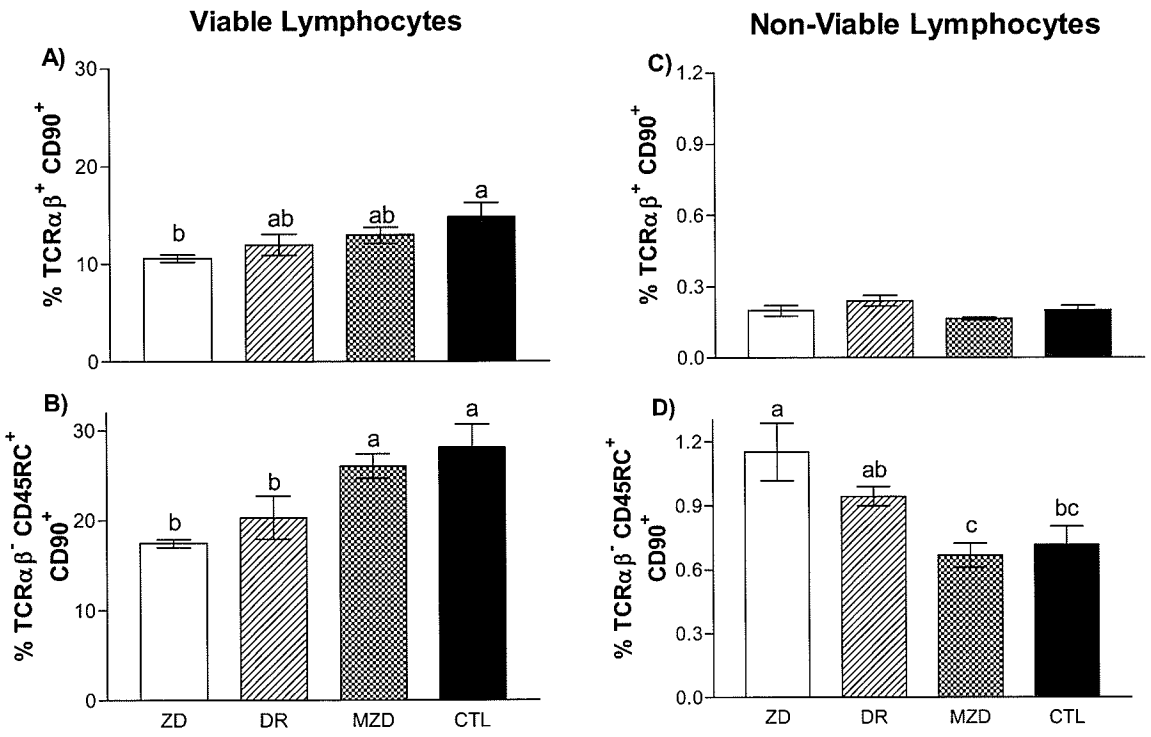


Figure 7.7.4. Proportion of splenic $CD90^+$ T- and B- lymphocytes (viable and non-viable) from zinc-deficient, marginally zinc-deficient, diet-restricted and control rats. Proportion of $TCR\alpha\beta^+$ $CD90^+$ cells in lymphocyte gate [A]; Proportion of $TCR\alpha\beta^-$ $CD45RC^+$ $CD90^+$ cells in lymphocyte gate [B]; Proportion of $TCR\alpha\beta^+$ $CD90^+$ cells in non-viable gate [C]; Proportion of $TCR\alpha\beta^-$ $CD45RC^+$ $CD90^+$ cells in non-viable gate. Values are means ± SEM for n=6 rats. Different letters indicate significant differences among means ($p \leq 0.05$).

Abbreviations: ZD=zinc-deficient group; DR=diet-restricted group; MZD=marginally zinc-deficient group; CTL=control group.

7.8 Discussion

There were no differences among the dietary treatment groups in the proportion of the most recent thymic emigrants ($TCR\alpha\beta^+CD90^+CD45RC^-RT6.1^-$ and $TCR\alpha\beta^+CD90^+CD45RC^-RT6.1^+$ cells) (Table 7.6.2) suggesting that maturation within and exit from the thymus was not affected by zinc deficiency. The results confirm our previous report that ZD rats have a smaller proportion of immature splenic T-cells ($CD90^+TCR\alpha\beta^+$) compared to CTL (6), and show that the lower proportion of $CD90^+TCR\alpha\beta^+$ cells (Figure 7.7.4a) is specifically due to a lower proportion of the late thymic emigrants ($TCR\alpha\beta^+CD90^+CD45RC^+RT6.1^+$ cells)(Table 7.6.2). Although DR and MZD both had fewer late thymic emigrants compared to CTL (Table 7.6.2), their overall percentage of $CD90^+TCR\alpha\beta^+$ cells was not significantly different from CTL (Figure 7.7.4a). It is interesting to note that even though the MZD group had a similar feed intake and body weight as the CTL group, they had lower proportions of late thymic emigrants similar to the DR and ZD groups which weighed 50% less than MZD.

One possibility for the lower proportion of late thymic emigrants ($TCR\alpha\beta^+CD90^+CD45RC^+RT6.1^+$ cells) in ZD is that they are being preferentially removed by apoptosis. However, this study does not support that theory, because there was no difference in the proportion of late thymic emigrants in the non-viable cell region or in the viable:non-viable ratio among groups. The main advantage of using flow cytometry to measure cell death is that cell surface markers can be included so that the phenotype of the cells that are non-viable can also be determined. In the present study we used change in light scatter as

a marker of cell death; however, inclusion of DNA dyes (ie. DAPI) to identify cells in the sub G1 area of the cell cycle (DNA fragmentation) or annexin-V (binds to phosphatidylserine; externalization of phosphatidylserine is an early indicator of apoptosis) would provide additional markers to separate necrotic, apoptotic and viable cells (25). Future studies should use the more sensitive measures described in the previous sentence to measure apoptosis in this cell population before it is definitively ruled out as the mechanism behind the lower proportion of late thymic emigrants. The methods used in the present study also only offer a snapshot of the splenocytes at a given time; however, an examination of changes over time would be of interest to determine whether the kinetics of cell death are altered by dietary zinc deficiency.

We believe that another possible explanation for the lower proportion of late thymic emigrants is a reduced ability of these cells to proliferate. $CD45RC^{+}$ cells produce IL-2, which stimulates cells to proliferate (26). The lower proportion of $CD45RC^{+}$ cells in ZD, DR, and MZD might lead to decreased IL-2 production by splenic T-cells, and therefore, less stimulation for these cells to proliferate, thereby lowering their proportions compared to CTL. Lowered production of IL-2 by peripheral blood mononuclear cells from zinc-deficient humans has been reported (27) and is consistent with this hypothesis. There is also evidence that the newly released T-cells respond to cytokine proliferation signals without any other stimulus and this response is lost as T-cells develop in the periphery (28) suggesting that late thymic emigrants would be more vulnerable to low IL-2 concentrations than the mature peripheral T-cells.

Another possible explanation for the lower proportion of late thymic emigrants is down regulation of the CD90 and/or CD45RC cell surface markers on these cells. CD90 is not only a maturational marker, but is involved in T-cell activation (29). CD45 is also involved in T-cell receptor signal transduction (13). Due to limiting resources in the zinc-deficient state, perhaps CD90 and/or CD45RC expression is down regulated in order to limit cellular activity and preserve resources.

In our previous work (6), we did see a decreased proportion of CD90+ T-cells in the blood and spleen of zinc deficient rats; however, the expression of CD90 in other peripheral lymphoid organs has not been explored in a model of dietary zinc deficiency. Perhaps in zinc deficiency the $CD90^{+}$ cells are homing to another peripheral lymphoid organ such as the lymph nodes or the Peyer's patches instead of the spleen. Future studies should include other peripheral lymphoid organs to address this issue.

In humans, there is currently no cell surface marker to identify recently released thymocytes in the periphery, thus researchers often rely on markers for naïve (CD45RA in humans, CD45RB in mice, and CD45RC in rats) versus memory T-cells (CD45RO in humans, $CD45RB^{-}$ in mice and $CD45RC^{-}$ in rats) (30). However, there is some controversy as to whether the CD45RA (or RB or RC) population is truly naïve. There is evidence that when truly naïve T-cells are exposed to antigen they express the lower molecular weight isoform of CD45, but they revert back to CD45RA (or RB or RC) if no antigen is present (17). Beck and colleagues (27) found that zinc-deficient humans had a trend ($p=0.077$)

towards a reduction in the ratio of naïve helper T-cells to memory helper T-cells and this suggested that zinc might be necessary for the generation of new T-cells. However, these labels can not determine whether there are fewer new T-cells exiting the thymus or fewer memory T-cells reverting back into naïve T-cells. The rat model offers the unique opportunity of being able to identify recently released thymocytes in the periphery using CD90 expression (7). ZD rats have fewer splenic $CD90^+$ T-cells (Figure 7.7.4a), however, by including CD45RC and RT6.1 labels we were able to determine that proportionally the generation of new T-cells by the thymus does not appear to be affected (no difference in the proportion of the most recently released thymocytes)(Table 7.6.2). The authors acknowledge that the ZD rat does have fewer total T-cells compared to CTL based on lymphoid organ size, and therefore, absolute number of T-cells generated by the ZD thymus is undoubtedly lower than the CTL thymus, however, the proportion of the newest splenic T-cell phenotypes is maintained. The ability to revert back into $CD45RC^+$ T-cells also does not appear to be affected, because there were no differences among the dietary treatment groups in the proportion of mature $CD45RC^-$ T-cells (Table 7.6.2).

We also report for the first time that ZD and DR have a lower proportion of immature B-cells ($TCR\alpha\beta^-CD45RC^+CD90^+$) in the spleen of the growing rat (Figure 7.7.6b). These cells also represented a higher proportion of cells in the non-viable cell region in ZD compared to CTL and MZD, which provides a possible explanation for their reduced proportion in the viable gate. It remains to be seen if the lower proportion of immature B-cells is also due in part to

decreased lymphopoiesis in the bone marrow of growing ZD and DR rats or solely post-marrow events. In the bone marrow of zinc-deficient adult mice, reports have shown a lower proportion of pre B-cells ($CD45R^{+}CD43^{-}IgM^{-}$), but not the immature plus mature B-cells ($CD45R^{+}CD43^{-}IgM^{+}$) (20).

We found a higher proportion of granulocytes in ZD, which is consistent with findings by King and Fraker (20) who reported increased proportions of granulocyte precursors in the bone marrow of adult zinc-deficient mice. It is also interesting to note that the higher proportion of granulocytes is only seen in the ZD group and not in the DR or MZD groups indicating that reduced feed intake alone or a marginal deficiency of zinc is not sufficient to increase the proportion of granulocytes. It has been suggested that the elevated serum corticosterone concentrations associated with ZD leave lymphocytes more susceptible to apoptosis while extending the half life of granulocytes (20). However, in the present study, we did not find evidence of increased splenocyte susceptibility to cell death and the proportion of splenic granulocytes in the DR group was not greater despite the fact that serum corticosterone levels in this condition are similar to those in ZD (31).

The results in this study indicate that ZD, DR and MZD are characterized by fewer late thymic emigrants, while ZD and DR also have fewer immature B-cells compared to CTL. These results are relevant to the longstanding hypothesis that the immunodeficiency associated with dietary zinc deficiency is attributable, at least in part, to imbalances among subsets of splenic lymphocytes. The lower proportion of late thymic emigrants and immature B-

cells in the spleens of zinc deficient and energy restricted rats suggests that there are fewer new T- and B-cells that are able to enter the mature lymphocyte pool. If there are fewer new cells entering the mature lymphocyte pool, then over time the lymphocyte repertoire will become limited, potentially altering T- and B-cell function and the ability of the whole organism to fight off disease (32).

7.9 Literature Cited

1. Hulsewe KW, van Acker BA, von Meyenfeldt MF, Soeters PB. Nutritional depletion and dietary manipulation: Effects on the immune response. World J Surg. 1999 Jun;23(6):536-44.

2. Mackall CL, Hakim FT, Gress RE. T-cell regeneration: All repertoires are not created equal. Immunol Today. 1997 May;18(5):245-51.

3. Berzins SP, Boyd RL, Miller JF. The role of the thymus and recent thymic migrants in the maintenance of the adult peripheral lymphocyte pool. J Exp Med. 1998 Jun 1;187(11):1839-48.

4. Baumann S, Krueger A, Kirchhoff S, Krammer PH. Regulation of T cell apoptosis during the immune response. Curr Mol Med. 2002 May;2(3):257-72.

5. Fischer Walker C, Black RE. Zinc and the risk for infectious disease. Annu Rev Nutr. 2004;24:255-75.

6. Hosea HJ, Rector ES, Taylor CG. Zinc-deficient rats have fewer recent thymic emigrant (CD90+) T lymphocytes in spleen and blood. J Nutr. 2003 Dec;133(12):4239-42.

7. Hosseinzadeh H, Goldschneider I. Recent thymic emigrants in the rat express a unique antigenic phenotype and undergo post-thymic maturation in peripheral lymphoid tissues. J Immunol. 1993 Mar 1;150(5):1670-9.

8. Kampinga J, Groen H, Klatter FA, Pater JM, van Petersen AS, Roser B, Nieuwenhuis P, Aspinall R. Post-thymic T-cell development in the rat. Thymus. 1997;24(3):173-200.

9. Mojcik CF, Greiner DL, Medlock ES, Komschlies KL, Goldschneider I. Characterization of RT6 bearing rat lymphocytes. I. ontogeny of the RT6+ subset. Cell Immunol. 1988 Jul;114(2):336-46.

10. Greiner DL, Reynolds GW, Lubaroff DM. Maturation of functional T-lymphocyte subpopulations in the rat. Thymus. 1982 Feb;4(2):77-90.

11. Fowell D, Mason D. Evidence that the T cell repertoire of normal rats contains cells with the potential to cause diabetes. characterization of the CD4+ T cell subset that inhibits this autoimmune potential. J Exp Med. 1993 Mar 1;177(3):627-36.

12. Thomas ML. The leukocyte common antigen family. Annu Rev Immunol. 1989;7:339-69.

13. Sasaki T, Sasaki-Irie J, Penninger JM. New insights into the transmembrane protein tyrosine phosphatase CD45. Int J Biochem Cell Biol. 2001 Nov;33(11):1041-6.

14. Xystrakis E, Cavailles P, Dejean AS, Cautain B, Colacios C, Lagrange D, van de Gaar MJ, Bernard I, Gonzalez-Dunia D, et al. Functional and genetic analysis of two CD8 T cell subsets defined by the level of CD45RC expression in the rat. J Immunol. 2004 Sep 1;173(5):3140-7.

15. Xystrakis E, Bernard I, Dejean AS, Alsaati T, Druet P, Saoudi A. Alloreactive CD4 T lymphocytes responsible for acute and chronic graft-versus-host disease are contained within the CD45RChigh but not the CD45RClow subset. Eur J Immunol. 2004 Feb;34(2):408-17.

16. Yoon JW, Jun HS. Viruses in type 1 diabetes: Brief review. ILAR J. 2004;45(3):343-8.

17. Bunce C, Bell EB. CD45RC isoforms define two types of CD4 memory T cells, one of which depends on persisting antigen. J Exp Med. 1997 Feb 17;185(4):767-76.

18. Fraker PJ. Roles for cell death in zinc deficiency. J Nutr. 2005 Mar;135(3):359-62.

19. King LE, Osati-Ashtiani F, Fraker PJ. Apoptosis plays a distinct role in the loss of precursor lymphocytes during zinc deficiency in mice. J Nutr. 2002 May;132(5):974-9.

20. King LE, Fraker PJ. Zinc deficiency in mice alters myelopoiesis and hematopoiesis. J Nutr. 2002 Nov;132(11):3301-7.

21. Iwakoshi NN, Goldschneider I, Tausche F, Mordes JP, Rossini AA, Greiner DL. High frequency apoptosis of recent thymic emigrants in the liver of lymphopenic diabetes-prone BioBreeding rats. J Immunol. 1998 Jun 15;160(12):5838-50.

22. Fraker PJ, King LE, Lill-Elghanian D, Telford WG. Quantification of apoptotic events in pure and heterogeneous populations of cells using the flow cytometer. Methods Cell Biol. 1995;46:57-76.

23. Hosea HJ, Taylor CG, Wood T, Mollard R, Weiler HA. Zinc-deficient rats have more limited bone recovery during repletion than diet-restricted rats. Exp Biol Med (Maywood). 2004 Apr;229(4):303-11.

24. Lepage LM, Giesbrecht JA, Taylor CG. Expression of T lymphocyte p56(lck), a zinc-finger signal transduction protein, is elevated by dietary zinc deficiency and diet restriction in mice. J Nutr. 1999 Mar;129(3):620-7.

25. Darzynkiewicz Z, Bedner E, Smolewski P. Flow cytometry in analysis of cell cycle and apoptosis. Semin Hematol. 2001 Apr;38(2):179-93.

26. Fowell D, McKnight AJ, Powrie F, Dyke R, Mason D. Subsets of CD4+ T cells and their roles in the induction and prevention of autoimmunity. Immunol Rev. 1991 Oct;123:37-64.

27. Beck FW, Prasad AS, Kaplan J, Fitzgerald JT, Brewer GJ. Changes in cytokine production and T cell subpopulations in experimentally induced zinc-deficient humans. Am J Physiol. 1997 Jun;272(6 Pt 1):E1002-7.

28. Hassan J, Reen DJ. Human recent thymic emigrants--identification, expansion, and survival characteristics. J Immunol. 2001 Aug 15;167(4):1970-6.

29. Loertscher R, Lavery P. The role of glycosyl phosphatidyl inositol (GPI)-anchored cell surface proteins in T-cell activation. Transpl Immunol. 2002 May;9(2-4):93-6.

30. Bell EB, Sparshott SM, Bunce C. CD4+ T-cell memory, CD45R subsets and the persistence of antigen--a unifying concept. Immunol Today. 1998 Feb;19(2):60-4.

31. Hosea HJ, Rector ES, Taylor CG. Dietary repletion can replenish reduced T cell subset numbers and lymphoid organ weight in zinc-deficient and energy-restricted rats. Br J Nutr. 2004 May;91(5):741-7.

32. La Gruta NL, Driel IR, Gleeson PA. Peripheral T cell expansion in lymphopenic mice results in a restricted T cell repertoire. Eur J Immunol. 2000 Dec;30(12):3380-6.

8. THE REDUCED PROPORTION OF NEW SPLENIC T-CELLS IN THE ZINC-DEFICIENT GROWING RAT IS NOT DUE TO INCREASED SUSCEPTIBILITY TO APOPTOSIS

Heather J. Hosea[*], Edward S. Rector[†], and Carla G. Taylor[*]

Departments of Human Nutritional Sciences[*] and Immunology[†]

8.1 Abstract

Dietary zinc deficiency has been associated with an increased risk of infection; however, an explanation for the immunodeficiency is not yet known. It has been reported that zinc-deficient rats have fewer New T-cells ($TCR\alpha\beta^{+}CD90^{+}$) compared to diet-restricted and control rats, which over time could adversely affect the ability of the organism to fight off infections. We hypothesized that the lower proportion of New T-cells in zinc deficiency is due to an increased susceptibility to apoptosis. Weanling, Sprague Dawley rats were assigned to one of four dietary treatment groups for 3 weeks: zinc-deficient (ZD, <1 mg zinc/kg, *ad libitum*), diet-restricted (DR, 30 mg zinc/kg, limited to the amount of feed as consumed by ZD), marginally zinc-deficient (MZD, 10 mg zinc/kg, *ad libitum*) or control (CTL, 30 mg zinc/kg, *ad libitum*). Thymocytes and splenocytes were labeled for flow cytometric determination of cell surface markers and DNA staining which allows for the simultaneous determination of the phenotype of apoptotic cells. There was no difference in the proportion of $CD90^{+}$ thymocytes; however, ZD rats had a higher proportion of Pro-T ($CD90^{+}4^{-}8^{-}$) and Cytotoxic ($CD90^{+}4^{-}8^{+}$) thymocytes compared to MZD and CTL. There was no effect of diet on the proportion of apoptotic thymocytes. ZD had a lower proportion of splenic New T-cells compared to DR, MZD and CTL, but there was no effect of diet on the proportion of apoptotic splenocytes. We characterized the splenic New T-cells into Helper and Cytotoxic subsets and found that ZD had a higher ratio of Helper to Cytotoxic New T-cells compared to MZD and CTL. These results do not support the hypothesis of increased apoptotic removal of

New T-cells in ZD. The regulation of CD90 expression should be explored in future studies.

8.2 Introduction

It has been estimated that zinc deficiency affects 1/3 of the world's population with children being a segment of the population at particular risk (1). Zinc deficiency has been known to impair immune function, and is the cause of approximately 10% of diarrheoal disease, 16% of lower respiratory tract infections, and 18% of malaria cases (1). Studies from around the world have shown that zinc supplementation reduces the rates of diarrhea, pneumonia, malaria and mortality in children (2-5). It has therefore, been of great interest to determine why dietary zinc deficiency leaves children more susceptible to disease.

Studies of zinc-deficient adult mice have shown lymphoid organ atrophy, lower T-cell numbers, impaired T-cell function and a decreased ability to fight infection compared to control mice (6). Elevated corticosterone levels have also been associated with increased apoptosis of Pre-T cells ($CD4^+CD8^+$) and greatly reduced thymocyte numbers in zinc-deficient adult mice (7). King and colleagues (7) postulate that these results explain the lymphopenia and subsequent increased vulnerability to disease in dietary zinc deficiency.

Lepage and colleagues (8) and Moore and colleagues (9) reported elevated splenocyte and thymocyte $p56^{lck}$ protein levels in the zinc-deficient mouse. $P56^{lck}$ is a signaling protein which is necessary for the maturation of thymocytes and is involved in the signaling pathway which initiates apoptosis (10-12). Research using the zinc-deficient growing rat did not uncover any change in the phenotypic distribution of T-cells from the thymus, spleen and

blood, with the exception of fewer $CD90^+$ T-lymphocytes in the blood and spleen, but not thymus of zinc deficient rats, compared to diet restricted and control rats (13). CD90 first appears on cortical thymocytes and is found on newly released T-cells (recent thymic emigrants) for approximately 3 days in rats (14). A greater susceptibility of recent thymic emigrants to apoptosis has been reported in the diabetes-prone BB rat (15), which are similar to zinc deficient rats in that they have reduced lymphocyte numbers compared to controls, and the proportion of T-cell subsets using CD4 and CD8 labeling remains unchanged (16).

We hypothesized that the reduced proportion of recent thymic emigrant T-cells in zinc-deficient rats is due to their increased susceptibility to apoptosis upon exiting the thymus. Flow cytometry can be used to quantify apoptosis in specific cell populations by simultaneously staining cell surface markers and DNA to generate cell cycle profiles (17). The cells that appear in the sub G1 area of the cell cycle are apoptotic. In the present experiment, we used flow cytometry and four or five-color labeling of thymic and splenic cells for characterization of T-cell phenotypes and to determine proportion of apoptosis in T-cell sub-populations among zinc-deficient, diet-restricted, marginally zinc-deficient and control growing rats.

8.3 Materials and Methods

8.8.1 Animals and diet

Weanling Sprague Dawley rats (Charles River Laboratories, St. Constant, PQ) were acclimatized for 5 days and randomly assigned to one of four dietary

treatment groups for three weeks: zinc-deficient (ZD, <1 mg zinc/kg diet), marginally zinc-deficient (MZD, 10 mg zinc/kg diet), nutritionally complete control diet (CTL, 30 mg zinc/kg diet), and diet-restricted (fed the control diet, but only the amount of feed as consumed on the previous day by the individual zinc deficient rat paired to the DR rat). The diet-restricted group controls for the effects of undernutrition commonly seen in zinc-deficiency. Body weight was measured weekly and feed intake was measured daily.

The experimental diets (based on the American Institute of Nutrition-93G formulation and previously described by Lepage et al, (8)) containing egg white, additional biotin (2 mg/kg diet) and potassium phosphate were fed *ad libitum*, except for the DR group, and distilled water was provided. Zinc content of the diets was verified by atomic absorption analysis. To avoid zinc recycling and contamination, the rats were housed individually in stainless steal hanging cages with mesh bottoms so urine and feces could drop to the shavings below. In addition, the zinc-deficient group was placed on the upper rows of the cage rack. The rats were maintained in an environment of controlled temperature (21-23°C), humidity (55%) and light cycle (14 hours light/10 hours dark). Animal care was provided in accordance with a protocol approved by the University of Manitoba Protocol Management and Review Committee.

8.3.2 Sample collection

At the end of the feeding trial, the rats were euthanized by CO_2 asphyxiation and decapitation. Trunk blood was collected and centrifuged to obtain serum and stored at -80°C until analysis. The spleen and thymus were

removed aseptically, weighed and processed immediately. Femurs were removed, cleaned of soft tissue and stored at -20°C until analysis.

Cell Preparation

Single cell suspensions of spleen and thymus were prepared by pressing tissues through nylon screens into Hank's buffered saline supplemented with 10 mM HEPES, 4% dextran charcoal absorbed fetal bovine serum (Hyclone, Logan, UT), and 1% antibiotic/antimycotic at pH 7.4. Dextran charcoal absorbed fetal bovine serum was used to limit the amount of external steroids that the splenocytes are exposed to during cell preparation. Red blood cells from the spleen cell suspensions were lysed using ammonium chloride lysis buffer (155 mM NH_4Cl, 0.1 mM EDTA,10 mM $KHCO_3$, pH 7.2). Cell concentration and viability were determined using trypan blue dye exclusion on an AO Bright-Line Hemacytometer (American Optical Corporation, Buffalo, NY).

8.3.3 Outcome measurements

Zinc Analysis

After obtaining wet and dry weights, femurs and diet samples were wet-ashed using trace element grade nitric acid. After appropriate dilution of digests or serum, zinc concentration was determined by atomic absorption spectrometry (Varian Spectra AA, Varian Australia, Mulgrave, VI). Bovine liver standard reference material 1577b (US Department of Commerce, National Institute of Standards and Technology, Gaithersburg, MD) was used as quality control.

Determination of T-lymphocyte Subpopulations

Antibodies: Monoclonal antibodies for TCRαβ (PE label, clone R73, isotype mouse $IgG_{1,k}$), CD90 (FITC label, Thy1.1, clone OX-7, isotype mouse $IgG_{1,k}$), CD4 (PE-Cy5 label, clone OX-35, isotype mouse $IgG_{2a,k}$), and CD8 (biotin, clone OX-8, isotype mouse $IgG_{1,k}$) were obtained from BD Pharmingen (Mississauga, ON). Streptavidin-PE-Cy7 conjugate was used to label the biotinylated antibodies. DAPI (0.2 µmol/L; Sigma Chemical Company, St. Louis, MO) was used to stain DNA to generate cell cycle data.

Cell Labeling and Flow Cytometry: Because apoptotic cells are rapidly removed *in vivo* by phagocytes, it can be difficult to study apoptotic cells. Therefore, thymocytes and splenocytes were subjected to three *in vitro* conditions. We analyzed cells immediately (IMM) to determine the phenotype immediately upon cell isolation, or the cells were cultured for 7 h (INC) to allow the cells that were stimulated *in vivo* to undergo apoptosis to appear in the relative absence of phagocytes, or the cells were incubated for 7 h with dexamethasone (DEX) to initiate apoptosis and determine whether dietary treatment influenced the susceptibility of thymocytes and splenocytes to apoptosis *in vitro*. Cells (1×10^6) from each dietary treatment group and tissue were incubated with fluorescent antibodies immediately, while the remaining cells were resuspended in culture medium (RPMI-1640 containing 10 mM HEPES, 10 mM sodium bicarbonate, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 50 µM 2-mercaptoethanol, and 5% fetal bovine serum (Sigma, St. Louis, MO)) and cultured for 7 h at 37°C with 5% CO_2 in the absence or presence of 1 µmol/L

dexamethasone. The dexamethasone-treated cells served as a positive control for apoptosis by creating a hypodiploid population for DNA analysis on the flow cytometer.

Either immediately after isolation or after the culture period, cells were washed and placed in label buffer (phosphate buffered saline containing 23 mM sodium azide and 2% fetal bovine serum, pH 7.4) at 4°C at a concentration of 1 x 10^6 cells/mL. The biotinylated anti-CD8 antibody was added first, incubated for 30 minutes at 4°C, followed by a wash step. Then the directly conjugated antibodies and streptavidin-PE-Cy7 were added, incubated as previously described, followed by a wash step. Samples were resuspended in phosphate buffered saline containing 50% fetal bovine serum. Ethanol (70%) was added dropwise while gently mixing to a final concentration of 50%. Cells were stored overnight at 4°C. The following morning, cells were washed and resuspended in 1 mL phosphate buffered saline. DAPI (final concentration=0.1µg/mL) was added and cells were analyzed on flow cytometer.

Flow cytometry analysis was performed on a Beckman Coulter EPICS ALTRA (Beckman Coulter, Mississauga, ON) high-speed cell sorter with laser excitation tuned to 488 nm (65 mW) and UV. Forward versus side scatter histograms were used to setup a wide gate, including both intact and apoptotic lymphoid cells. The fluorescence signals were separated with the standard dichotic long pass filters provided with the instrument and detected through 424 nm (DAPI), 525 nm (FITC), 575 nm (PE), 675 nm (PE-Cy5) and 735 nm (PE-Cy7) bandpass filters. The data was collected in listmode format with the

subsequent analyses based on 20 000 cells satisfying the light scatter gate for $CD90^+$ in thymocytes, or 20 000 cells satisfying the light scatter gate for $TCR\alpha\beta^+CD90^-$ in splenocytes using the EXPO32 MultiCOMP MFA software provided with the instrument. Flurochrome-isotype matched controls were prepared to assess autofluorescence and nonspecific binding, while single color samples were employed to adjust color compensation. Representative flow plots from thymocytes and splenocytes are shown in Figures 8.7.1-8.7.4.

8.4 Statistical Analyses

Body weight, feed intake, lymphoid organ weight, serum and femur zinc concentration data were analyzed by one-way ANOVA, lymphocyte phenotype data was analyzed by two-way ANOVA (main effects diet and treatment) and cell cycle data was analyzed by three-way ANOVA (main effects diet, treatment and cell type) using the general linear models procedure (SAS software release 8.2, SAS Institute, Cary NC). Significant differences among means were determined using Duncan's multiple range test. Differences were considered significant at $p<0.05$.

8.5 Results

Body weight, feed intake, lymphoid organ weight and zinc status. Body weight and feed intake of ZD was 49% less than MZD and CTL (Table 8.6.1). Despite consuming the same amount of food, ZD weighed 15% less than DR. There were no differences among dietary treatment groups in lymphoid organ weight

when it was corrected for body weight. Dietary zinc levels were reflected in serum and femur zinc concentrations.

Thymocyte subsets. Thymocytes mature from Pro-T ($CD90^+CD4^-CD8^-$) into Pre-T ($CD90^+CD4^+CD8^+$) and then become either Helper ($CD90^+CD4^+CD8^-$) or Cytotoxic ($CD90^+CD4^-CD8^+$) T-cells before leaving the thymus (7). There were no differences among the dietary treatment groups in the total proportion of thymocytes expressing CD90 or in Pre-T and Helper thymocytes (Table 8.6.2). ZD rats had a trend towards a higher proportion of Pro-T ($p=0.0560$) and a 21% higher proportion of Cytotoxic thymocytes ($p=0.0191$) compared to MZD and CTL, but not DR. There was no difference in the ratio of Helper to Cytotoxic thymocytes. *In vitro* treatment of the cells had no effect on the proportion of $CD90^+$, Pre-T and Helper thymocytes. However, there was a 19% higher proportion of Pro-T thymocytes in the IMM group compared to INC and DEX, while there was a 18% higher proportion of Cytotoxic thymocytes in the DEX treated groups compared to IMM and INC. There was a 28% higher ratio of Helper to Cytotoxic thymocytes in the IMM group compared to INC and DEX. There was no interaction between diet and *in vitro* treatment.

Apoptotic thymocytes. There was an interaction between cell type and *in vitro* treatment (Figure 8.7.5a). In general, IMM had the lowest proportion, INC had an intermediate proportion, and DEX had the highest proportion of apoptotic thymocytes. When thymocytes were analyzed immediately, Pro-T cells represented a higher proportion of cells in the apoptotic region compared to Pre-T-cells. After incubation for 7 h, Cytotoxic thymocytes represented the highest

proportion of cells in the apoptotic region, followed by the Pro-T cells while the Pre-T and Helper thymocytes represented the lowest proportions. After incubation for 7 h with dexamethasone, the Cytotoxic thymocytes represented the highest proportion of cells in the apoptotic region while the Helper thymocytes represented the lowest proportion of apoptotic cells. Dietary treatment did not affect the proportion of apoptotic thymocytes (Figure 8.7.5b).

Splenic T-cell subsets. ZD, DR and MZD had a 22% higher proportion of T-cells ($TCR\alpha\beta^+$) compared to CTL (Table 8.6.3). ZD had a 12-23% lower proportion of New T-cells ($TCR\alpha\beta^+CD90^+$) compared to DR, MZD and CTL due to a 10-17% lower proportion of New Helper ($TCR\alpha\beta^+CD90^+CD8^-CD4^+$) and a 13-25% lower proportion of New Cytotoxic T-cells ($TCR\alpha\beta^+CD90^+CD8^+CD4^-$). The higher ratio of New Helper to New Cytotoxic T-cells in ZD compared to MZD and CTL indicates that the New Cytotoxic T-cells were lowered more in relation to the New Helper T-cells. The proportion of Mature T-cells ($TCR\alpha\beta^+CD90^-$) was highest in ZD due to an 11% higher proportion of Mature Helper T-cells ($TCR\alpha\beta^+CD90^-CD8^-CD4^+$) compared to DR, MZD and CTL. DR had a 13% lower proportion of New T-cells compared to CTL, due to a lower proportion of New Cytotoxic T-cells. DR had a 2-4% higher proportion of Mature T-cells compared to MZD and CTL, but the ratio of Helper to Cytotoxic was not different. MZD had a 2% higher proportion of Mature T-cells, but not a lower proportion of New T-cells compared to CTL. There was no difference in the proportion of New or Mature Helper or Cytotoxic T-cells between MZD and CTL.

In vitro treatment of cells did not affect the proportion of Total T-cells, but IMM had a 23% lower proportion of New T-cells (11% fewer Helper and 17% fewer Cytotoxic) and a 4% higher proportion of Mature T-cells (12% more Helper, but no difference in Cytotoxic). There was no difference between INC and DEX in the proportion of any of the T-cell subsets studied. There was no interaction between diet and *in vitro* treatment.

Apoptotic splenocytes. When the proportion of New and Mature splenic T-cells were included in the 3-way ANOVA, there was no effect of diet on the proportion of apoptotic splenic T-cells (Figure 8.7.6). There was an effect of *in vitro* treatment where DEX had a 61% higher proportion of apoptotic splenocytes compared to INC, and INC had a 718% higher proportion of apoptotic splenocytes compared to IMM. Cell type had an effect on the proportion of apoptotic splenocytes where New splenic T-cells had an 8% higher proportion of cells in the apoptotic region compared to Mature splenic T-cells.

When the splenic T-cells were further divided into New and Mature Cytotoxic and Helper T-cells (Figure 8.7.7), the 3-way ANOVA revealed a trend towards an effect of diet ($p=0.0597$), an effect of treatment ($p<0.0001$), cell type ($p=0.0003$) and an interaction between treatment and cell type ($p=0.0035$). As with the thymocytes, IMM had the lowest proportion of splenocytes in the apoptotic region, while INC had an intermediate proportion and DEX had the highest proportion of splenocytes in the apoptotic region (Figure 8.7.7a). In the IMM group, the cell types were equally represented in the apoptotic region, but in the INC group the New Helper T-cells were at a lower proportion compared to

New Cytotoxic and Mature Cytotoxic. In the DEX group the New Cytotoxic T-cells represented the highest proportion of cells in the apoptotic region. ZD and MZD had a 9% lower proportion of apoptotic splenocytes compared to CTL but they were not different from DR (Figure 8.7.7b).

8.6 Tables

Table 8.6.1. Body weight, feed intake, lymphoid organ weights and zinc status of zinc-deficient, marginally zinc-deficient, diet-restricted, and control rats[1].

	Dietary Group[2]			
	ZD	DR	MZD	CTL
Final body weight (g)	140 ± 4.8^{c}	164 ± 5.0^{b}	276 ± 4.0^{a}	278 ± 4.1^{a}
Total feed intake (g)	216 ± 7.1^{b}	222 ± 7.3^{b}	411 ± 8.1^{a}	420 ± 16^{a}
Thymus(mg):bwt(g)	3.0 ± 0.1	3.2 ± 0.1	3.2 ± 0.1	3.4 ± 0.1
Spleen:body weight	2.7 ± 0.1	2.5 ± 0.1	2.5 ± 0.1	2.7 ± 0.1
Serum zinc (µmol/L)	5.2 ± 0.3^{d}	26.2 ± 1.2^{b}	18.5 ± 0.9^{c}	30.6 ± 1.3^{a}
Femur zinc (µmol zinc/g dry bone)	1.04 ± 0.05^{d}	4.08 ± 0.10^{b}	3.01 ± 0.12^{c}	4.82 ± 0.13^{a}

[1]Values are means ± SEM, n=8. Different superscript letters indicate significant differences among means, $p < 0.05$.

[2]ZD=zinc-deficient group; DR=diet-restricted group; MZD=marginally zinc-deficient group; CTL=control group.

Table 8.6.2. The effect of diet and *in vitro* treatment on the proportion of thymocyte subsets[1].

	Dietary Treatment[2]					*In vitro* Treatment[3]			
	ZD	DR	MZD	CTL	p-value	IMM	INC	DEX	p-value
Total CD90$^+$	95.3±0.7	95.1±0.8	95.2±0.9	94.8±1.0	0.9777	95.0±0.6	94.7±0.9	95.5±0.7	0.7606
Pro-T	3.2±0.2^a	3.1±0.2^{ab}	2.7±0.2^b	2.6±0.2^b	0.0560	3.3±0.2*	2.7±0.1	2.8±0.2	0.0213
Pre-T	79.1±0.6	79.1±0.9	80.8±1.0	80.2±1.0	0.4832	79.4±0.9	80.5±0.8	79.5±0.7	0.5270
Helper	9.8±0.5	10.1±0.7	9.6±0.7	10.3±0.8	0.8812	11.0±0.7	9.5±0.5	9.4±0.5	0.1042
Cytotoxic	8.3±0.4^a	7.7±0.4^{ab}	6.9±0.4^b	6.8±0.3^b	0.0191	6.6±0.3*	7.3±0.3*	8.3±0.3	0.0028
Helper/Cytotoxic	1.2±0.1	1.4±0.1	1.4±0.1	1.5±0.1	0.1889	1.6±0.1*	1.3±0.1	1.2±0.1	0.0160

[1]As there was no interaction between diet and *in vitro* treatment but there was a main effect of diet and a main effect of *in vitro* treatment, data was pooled to show means of main effects only. Values are means ± SEM, n=24 (per diet) and n=32 (per *in vitro* treatment) with the following exceptions where data points were removed because they were greater than 3 standard deviations from the mean: n=23 for ZD Pre T-cell and n=31 for IMM Pre T-cell (data point value=55); n=23 for ZD Cytotoxic and n=31 for IMM Cytotoxic (data point value=21.6); n=23 for MZD and CTL Helper/Cytotoxic and n=30 for IMM Helper/Cytoxtic (data points values= 4.3 and 4.2, respectively).

[2]Different superscript letters indicate significant differences among dietary treatment means, $p < 0.05$.

[3]Asterisk indicates significantly different from DEX group means, $p<0.05$.

Abbreviations: ZD=zinc-deficient group; DR=diet-restricted group; MZD=marginally zinc-deficient group; CTL=control group; IMM=stained immediately; INC=incubated for 7h; DEX=incubated for 7h with dexamethasone; Pro-T=$CD90^{+}4^{-}8^{-}$; Pre-T=$CD90^{+}4^{+}8^{+}$; Helper= $CD90^{+}4^{+}8^{-}$; Cytotoxic= $CD90^{+}4^{-}8^{+}$.

Table 8.6.3. The effect of diet and *in vitro* treatment on the proportion of splenocyte subsets[1].

	Dietary Treatment[2]					*In vitro* Treatment[3]			
	ZD	DR	MZD	CTL	p-value	IMM	INC	DEX	p-value
Total T-cells	32.9±1.1[a]	35.1±1.1[a]	31.6±1.1[a]	27.2±1.3[b]	<0.0001	31.4±1.0	31.5±1.2	32.1±1.1	0.9061
New T-cells	11.4±0.4[c]	13.0±0.4[b]	14.2±0.5[ab]	14.9±0.7[a]	<0.0001	11.4±0.5*	13.9±0.4	14.8±0.4	<0.0001
New Helper	5.3±0.2[b]	5.9±0.2[a]	6.4±0.2[a]	6.3±0.2[a]	0.0007	5.6±0.2*	6.0±0.2	6.3±0.2	0.0212
New Cytotoxic	4.0±0.2[c]	4.6±0.1[b]	5.3±0.2[a]	5.3±0.2[a]	<0.0001	4.3±0.2*	4.9±0.2	5.2±0.2	0.0001
New Ratio	1.4±0.04[a]	1.3±0.04[ab]	1.2±0.04[b]	1.2±0.04[b]	0.0425	1.3±0.03*	1.3±0.04	1.2±0.04	0.0391
Mature T-cells	86.7±0.4[a]	85.1±0.5[b]	83.4±0.5[c]	82.1±0.7[d]	<0.0001	86.1±0.5*	83.8±0.5	83.0±0.5	<0.0001
Mature Helper	48.6±1.0[a]	44.6±1.3[b]	43.8±0.9[b]	43.1±1.4[b]	0.0041	48.0±0.8*	44.1±1.1	42.9±1.1	0.0009
Mature Cytotoxic	31.6±0.6	34.0±1.0	34.1±0.8	32.1±1.1	0.1461	32.8±0.7	33.1±0.9	32.9±0.9	0.9626
Mature Ratio	1.6±0.06	1.4±0.08	1.3±0.06	1.4±0.08	0.0870	1.5±0.06	1.4±0.07	1.4±0.07	0.1946

[1]As there was no interaction between diet and *in vitro* treatment but there was a main effect of diet and a main effect of *in vitro* treatment, data was pooled to show means of main effects only. Values are means ± SEM, n=24 (per diet) and n=32 (per *in vitro* treatment) with the following exceptions: n=23 for MZD Total T-cells and n=31 for IMM Total T-cells [data point (value=48.7) removed because it was greater than 3 standard deviations from the mean];

[2]Different superscript letters indicate significant differences among dietary treatment means, $p < 0.05$.

[3]Asterisk indicates significantly different from DEX group means, $p<0.05$.

Abbreviations: ZD=zinc-deficient group; DR=diet-restricted group; MZD=marginally zinc-deficient group; CTL=control group; IMM=stained immediately; INC=incubated for 7h; DEX=incubated for 7h with dexamethasone; Total T-cells= $TCR\alpha\beta^{+}$; New T-cells= $TCR\alpha\beta^{+}CD90^{+}$; New Helper= $TCR\alpha\beta^{+}CD90^{+}4^{+}8^{-}$; New Cytotoxic= $TCR\alpha\beta^{+}CD90^{+}4^{-}8^{+}$; New Ratio=New Helper/New Cytotoxic; Mature T-cells= $TCR\alpha\beta^{+}CD90^{-}$; Mature Helper= $TCR\alpha\beta^{+}CD90^{-}4^{+}8^{-}$; Mature Cytotoxic= $TCR\alpha\beta^{+}CD90^{+}4^{-}8^{+}$; Mature Ratio=Mature Helper/Mature Cytotoxic.

8.7 Figures

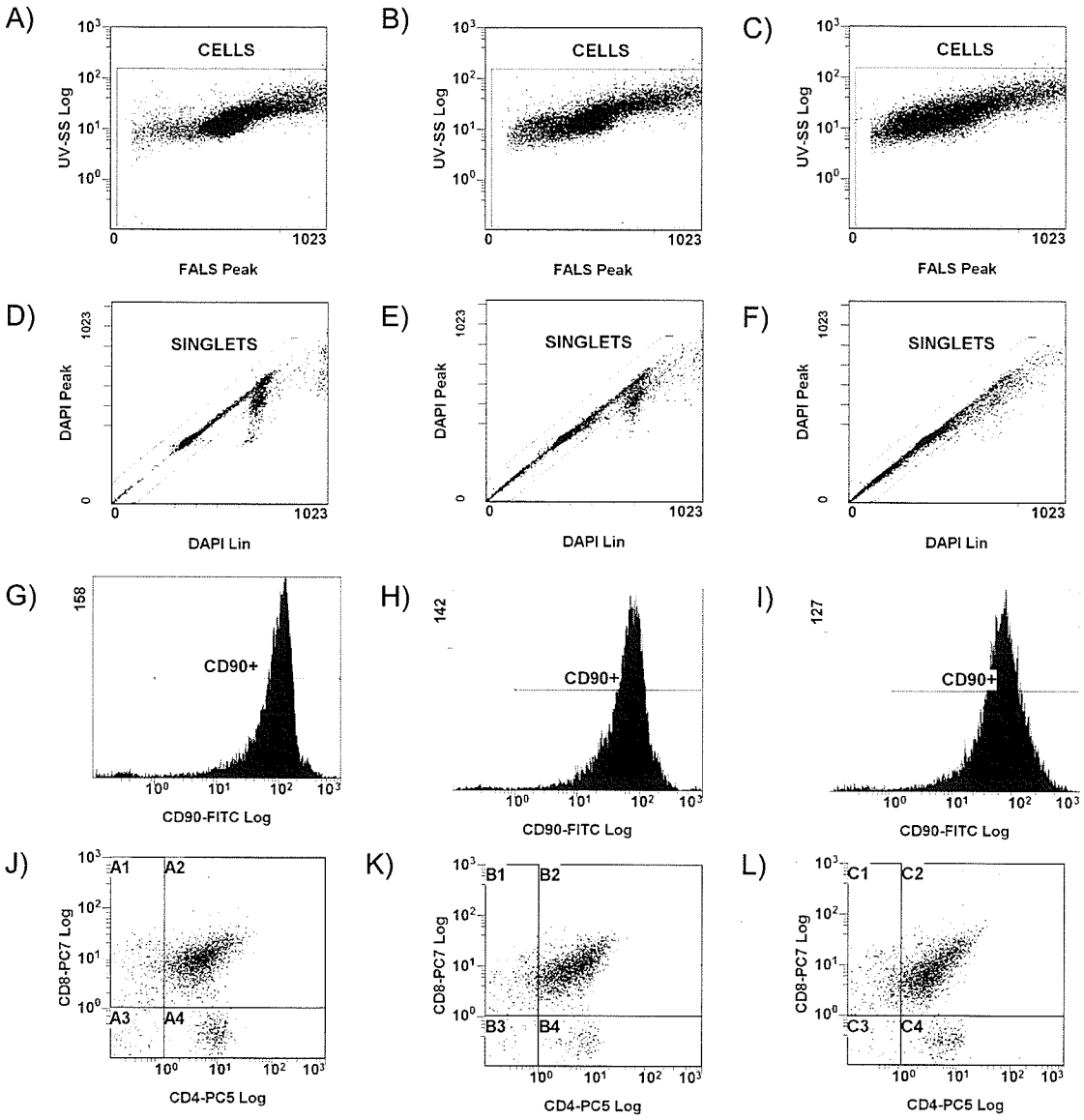


Figure 8.7.1. Representative flow cytometry plots of thymocytes either immediately (IMM), or after incubation for 7 h in cell culture media without (INC) or with dexamethasone (DEX). Definition of lymphocytes (CELLS) IMM [A], INC [B], and DEX [C]; Definition of singlets (eliminates cells that are stuck together) gated on CELLS IMM [D], INC [E], and DEX [F]; Definition of $CD90^+$ cells gated on CELLS and SINGLETS IMM [G], INC [H], and DEX [I]; Definition of CD8 and CD4 staining gated on CELLS, SINGLETS, and $CD90^+$ IMM [J], INC [K], and DEX [L]. IMM=stained immediately; INC=incubated for 7h; DEX=incubated for 7h with dex.

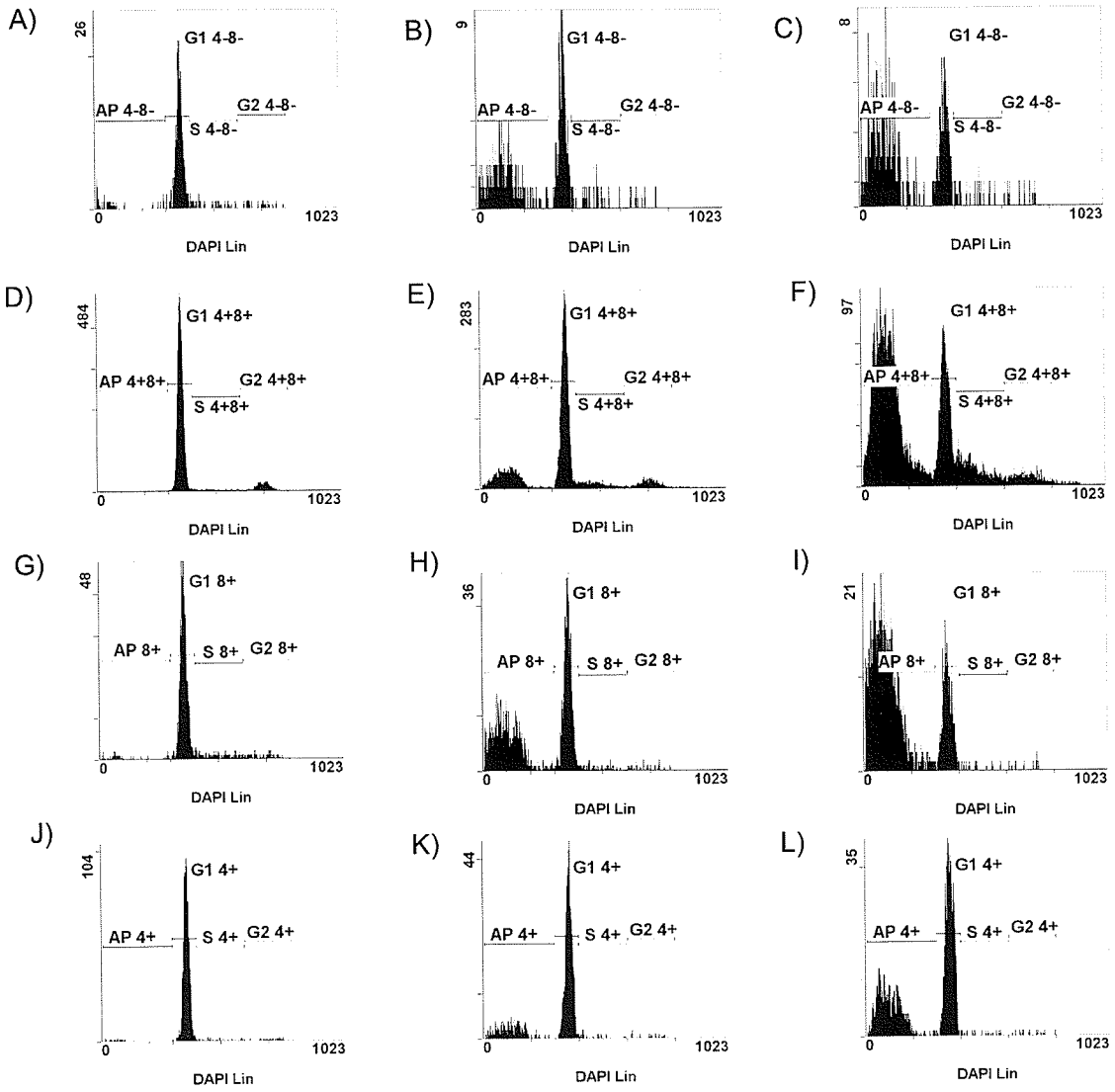


Figure 8.7.2. Representative cell cycle plots of thymocytes either immediately (IMM), or after incubation for 7 h in cell culture media without (INC) or with dexamethasone (DEX). Pro-T cell cycle IMM [A], INC [B], and DEX [C]; Pre-T cell cycle IMM [D], INC [E], and DEX [F]; Cytotoxic cell cycle IMM [G], INC [H], and DEX [I]; Helper cell cycle IMM [J], INC [K], and DEX [L].

Abbreviations: IMM=stained immediately; INC=incubated for 7h; DEX=incubated for 7h with dexamethasone; Pro-T=$CD90^{+}4^{-}8^{-}$; Pre-T=$CD90^{+}4^{+}8^{+}$; Cytotoxic= $CD90^{+}4^{-}8^{+}$; Helper= $CD90^{+}4^{+}8^{-}$.

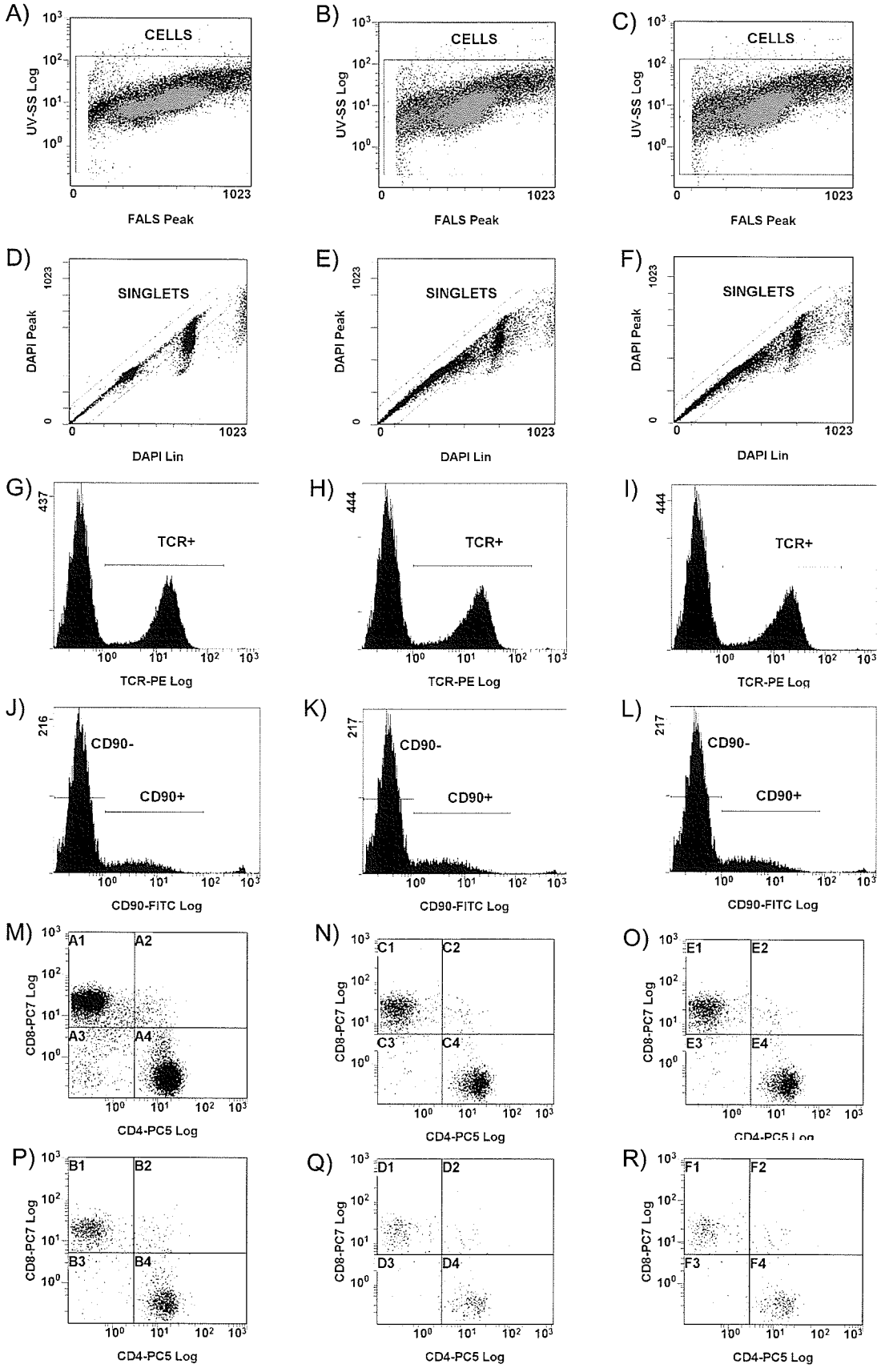

A)
CELLS
UV-SS Log
FALS Peak
B)
CELLS
UV-SS Log
FALS Peak
C)
CELLS
UV-SS Log
FALS Peak
D)
SINGLETS
DAPI Peak
DAPI Lin
E)
SINGLETS
DAPI Peak
DAPI Lin
F)
SINGLETS
DAPI Peak
DAPI Lin
G)
TCR+
TCR-PE Log
H)
TCR+
TCR-PE Log
I)
TCR+
TCR-PE Log
J)
CD90-
CD90+
CD90-FITC Log
K)
CD90-
CD90+
CD90-FITC Log
L)
CD90-
CD90+
CD90-FITC Log
M)
A1
A2
A3
A4
CD8-PC7 Log
CD4-PC5 Log
N)
C1
C2
C3
C4
CD8-PC7 Log
CD4-PC5 Log
O)
E1
E2
E3
E4
CD8-PC7 Log
P)
B1
B2
B3
B4
CD8-PC7 Log
CD4-PC5 Log
Q)
D1
D2
D3
D4
CD8-PC7 Log
CD4-PC5 Log
R)
F1
F2
F3
F4
CD8-PC7 Log
CD4-PC5 Log

Figure 8.7.3. Representative flow cytometry plots of splenocytes either immediately (IMM), or after incubation for 7 h in cell culture media without (INC) or with dexamethasone (DEX).

Definition of lymphocytes (CELLS) IMM [A], INC [B], and DEX [C]; Definition of singlets (eliminates cells that are stuck together) gated on CELLS IMM [D], INC [E], and DEX [F]; Definition of TCR$\alpha\beta^+$ cells gated on CELLS and SINGLETS IMM [G], INC [H], and DEX [I]; Definition of CD90$^+$ and CD90$^-$ cells gated on CELLS, SINGLETS and TCR$\alpha\beta^+$ IMM [J], INC [K], and DEX [L]; Definition of CD8 and CD4 staining gated on CELLS, SINGLETS, TCR$\alpha\beta^+$ and CD90$^-$ IMM [M], INC [N], and DEX [O]; Definition of CD8 and CD4 staining gated on CELLS, SINGLETS, TCR$\alpha\beta^+$ and CD90$^+$ IMM [P], INC [Q], and DEX [R].

Abbreviations: IMM=stained immediately; INC=incubated for 7h; DEX=incubated for 7h with dexamethasone.

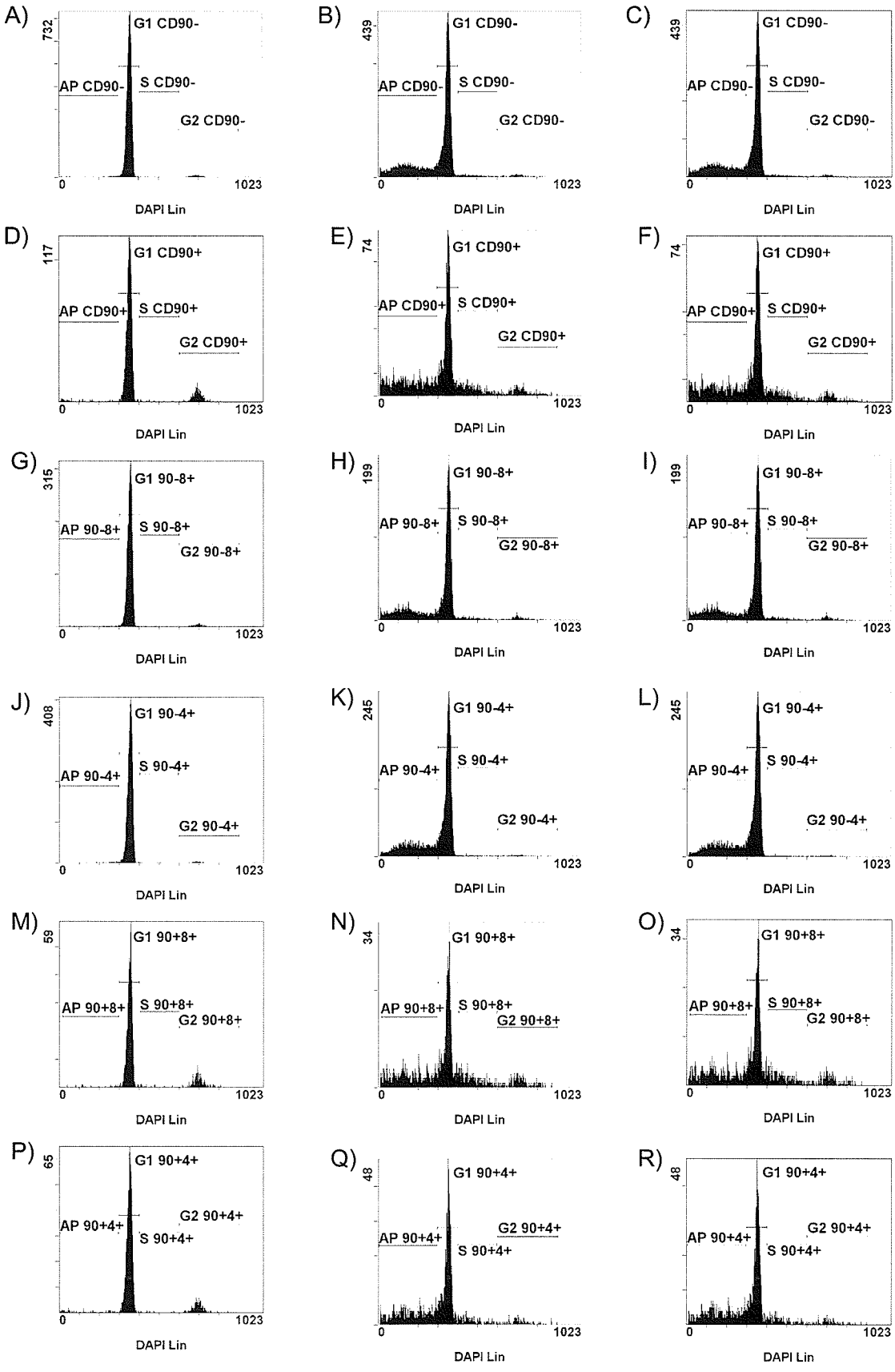

A)
732
G1 CD90-
AP CD90-
S CD90-
G2 CD90-
0
1023
DAPI Lin
B)
439
G1 CD90-
AP CD90-
S CD90-
G2 CD90-
0
1023
DAPI Lin
C)
439
G1 CD90-
AP CD90-
S CD90-
G2 CD90-
0
1023
DAPI Lin
D)
117
G1 CD90+
AP CD90+
S CD90+
G2 CD90+
0
1023
DAPI Lin
E)
74
G1 CD90+
AP CD90+
S CD90+
G2 CD90+
0
1023
DAPI Lin
F)
74
G1 CD90+
AP CD90+
S CD90+
G2 CD90+
0
1023
DAPI Lin
G)
315
G1 90-8+
AP 90-8+
S 90-8+
G2 90-8+
0
1023
DAPI Lin
H)
199
G1 90-8+
AP 90-8+
S 90-8+
G2 90-8+
0
1023
DAPI Lin
I)
199
G1 90-8+
AP 90-8+
S 90-8+
G2 90-8+
0
1023
DAPI Lin
J)
408
G1 90-4+
AP 90-4+
S 90-4+
G2 90-4+
0
1023
DAPI Lin
K)
245
G1 90-4+
AP 90-4+
S 90-4+
G2 90-4+
0
1023
DAPI Lin
L)
245
G1 90-4+
AP 90-4+
S 90-4+
G2 90-4+
0
1023
DAPI Lin
M)
59
G1 90+8+
AP 90+8+
S 90+8+
G2 90+8+
0
1023
DAPI Lin
N)
34
G1 90+8+
AP 90+8+
S 90+8+
G2 90+8+
0
1023
DAPI Lin
O)
34
G1 90+8+
AP 90+8+
S 90+8+
G2 90+8+
0
1023
DAPI Lin
P)
65
G1 90+4+
AP 90+4+
G2 90+4+
S 90+4+
0
1023
DAPI Lin
Q)
48
G1 90+4+
AP 90+4+
G2 90+4+
S 90+4+
0
1023
DAPI Lin
R)
48
G1 90+4+
AP 90+4+
G2 90+4+
S 90+4+
0
1023
DAPI Lin

Figure 8.7.4. Representative cell cycle plots of splenocytes either immediately (IMM), or after incubation for 7 h in cell culture media without (INC) or with dexamethasone (DEX).

Cell cycle of Mature T-cells IMM [A], INC [B], and DEX [C]; Cell cycle of New T-cells IMM [D], INC [E], and DEX [F]; Cell cycle of Mature Cytotoxic IMM [G], INC [H], and DEX [I]; Cell cycle of Mature Helper IMM [J], INC [K], and DEX [L]; Cell cycle of New Cytotoxic IMM [M], INC [N], and DEX [O]; Cell cycle of New Helper IMM [P], INC [Q], and DEX [R].

Abbreviations: IMM=stained immediately; INC=incubated for 7h; DEX=incubated for 7h with dexamethasone; Total T-cells= $TCR\alpha\beta^{+}$; New T-cells= $TCR\alpha\beta^{+}CD90^{+}$; New Cytotoxic= $TCR\alpha\beta^{+}CD90^{+}4^{-}8^{+}$; New Helper= $TCR\alpha\beta^{+}CD90^{+}4^{+}8^{-}$; Mature T-cells= $TCR\alpha\beta^{+}CD90^{-}$; Mature Cytotoxic= $TCR\alpha\beta^{+}CD90^{+}4^{-}8^{+}$; Mature Helper= $TCR\alpha\beta^{+}CD90^{-}4^{+}8^{-}$.

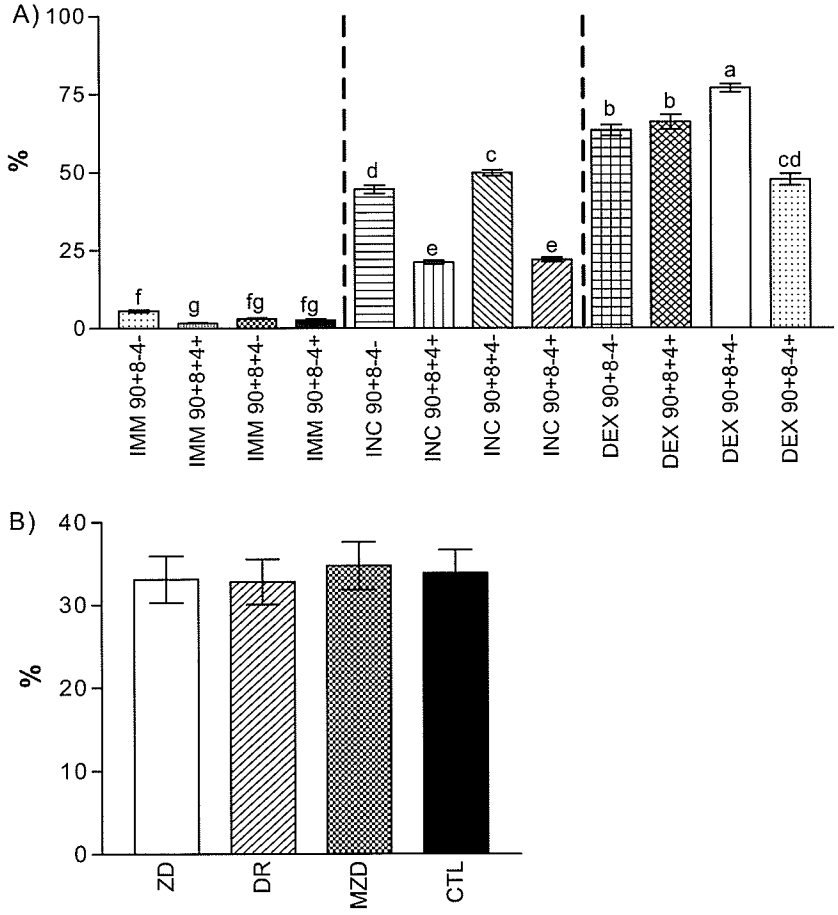


Figure 8.7.5. The proportion of apoptotic thymocyte subsets by diet, treatment and cell type. The statistical analysis (3-way ANOVA) showed main effects for cell type (P=<0.001), *in vitro* treatment (P=<0.0001), and interaction between cell type and *in vitro* treatment (P=<0.0001). Thus, the data was pooled to show the means for the interaction between cell type and treatment [A] expressed as mean ± standard error for n=32. Different superscript letters indicate statistical differences ($p<0.05$) among groups. There was no significant main effect of diet or significant interaction of diet with cell type or *in vitro* treatment; the diet means [B] are expressed as mean ± standard error for n=96.

Abbreviations: ZD=zinc-deficient; DR=diet-restricted; MZD=marginally zinc-deficient; CTL=control; IMM=stained immediately; INC=incubated for 7 hours; DEX=incubated for 7 hours with dexamethasone.

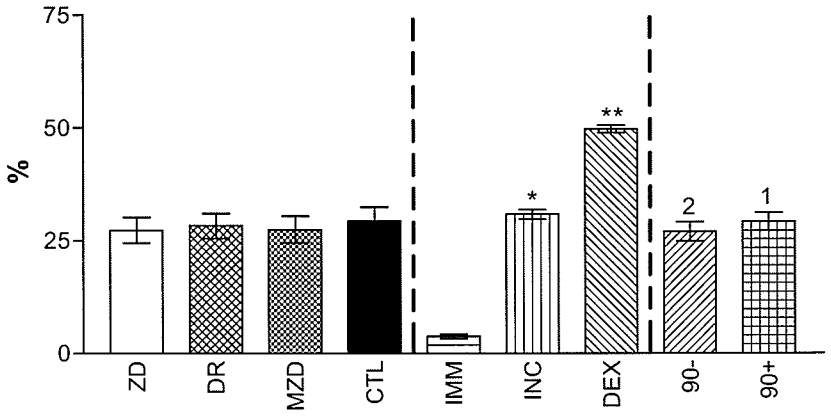


Figure 8.7.6. The proportion of apoptotic splenic subsets by diet, treatment and cell type for total CD90^{+} and CD90^{-} T-cells. The statistical analysis (3-way ANOVA) showed main effects for cell type (P=0.0246), and *in vitro* treatment (P=<0.0001), but no diet effect. Data expressed as mean ± standard error for n=48 for diet groups, n=64 for *in vitro* treatment groups and n=96 for cell type. * and ** indicates statistical differences ($p<0.05$) among *in vitro* treatment groups. Different superscript numbers indicate statistical differences ($p<0.05$) among cell types. Abbreviations: ZD=zinc-deficient; DR=diet-restricted; MZD=marginally zinc-deficient; CTL=control; IMM=stained immediately; INC=incubated for 7 hours; DEX=incubated for 7 hours with dexamethasone.

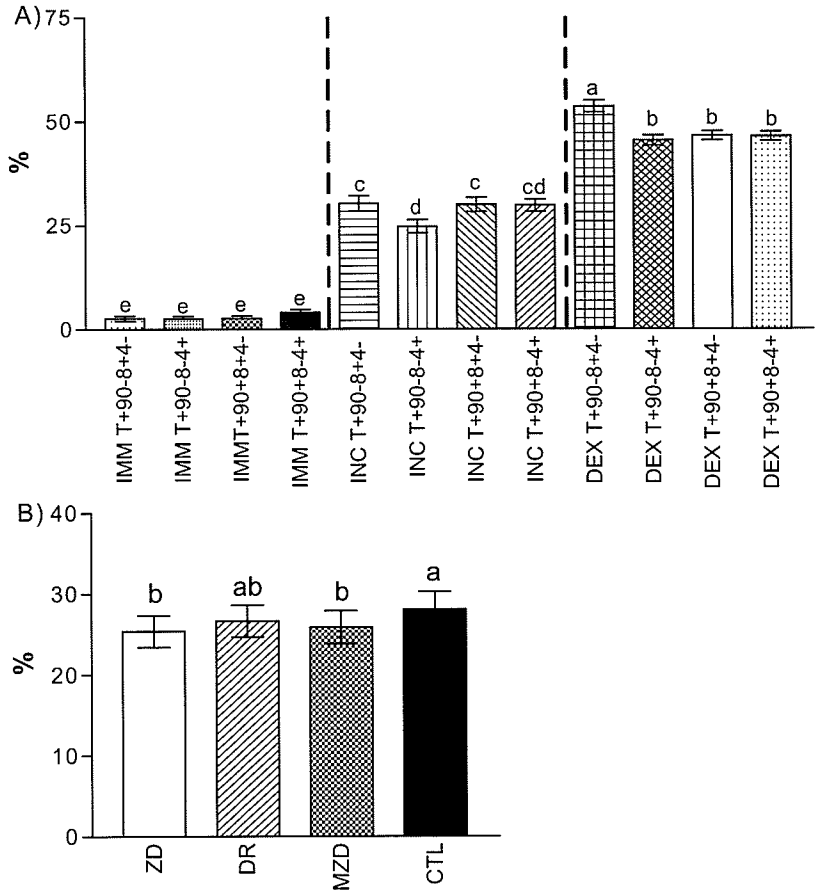


Figure 8.7.7. The proportion of apoptotic splenic subsets by diet, treatment and cell type for New and Mature Cytotoxic and Helper T-cells. The statistical analysis (3-way ANOVA) showed main effects for cell type (P=0.0003), *in vitro* treatment (P=<0.0001), and interaction between cell type and *in vitro* treatment (P=0.0035). Thus, the data was pooled to show the means for the interaction between cell type and treatment [A] expressed as mean ± standard error for n=32. There was a trend for the effect of diet (0.0597), but no significant interaction of diet with cell type or *in vitro* treatment; the diet means [B] are expressed as mean ± standard error for n=96. Different superscript letters indicate statistical differences ($p<0.05$) among groups. Abbreviations: ZD=zinc-deficient; DR=diet-restricted; MZD=marginally zinc-deficient; CTL=control; IMM=stained immediately; INC=incubated for 7 hours; DEX=incubated for 7 hours with dexamethasone.

8.8 Discussion

The rat offers the unique opportunity to study recent thymic emigrants, because unlike humans or mice, CD90 expression in the rat identifies newly released thymocytes. Dietary zinc deficiency in the growing rat results in fewer splenic New T-cells; however, the lower proportion in ZD is not due to an inability of the thymus to produce $CD90^+$ T-cells (Table 8.6.2) or increased susceptibility of $CD90^+$ T-cells to apoptosis in the thymus (Figure 8.7.5) or spleen (Figure 8.7.6) compared to DR, MZD and CTL. The susceptibility of splenic T-cells to apoptosis has not been explored previously in a model of dietary zinc deficiency; however, King and colleagues (7) reported opposite findings from thymocytes of zinc deficient adult mice (i.e. A threefold increase of apoptotic $CD4^+CD8^+$ thymocytes from ZD compared to CTL mice). Perhaps the discrepancy between the studies can be explained by an increased resiliency of the immune system to dietary stress in the young rat as opposed to the adult mouse.

This study is the first to characterize the CD4 and CD8 expression of splenic New T-cells in ZD. The altered ratio of New Helper to New Cytotoxic T-cells in ZD compared to MZD and CTL indicated a relative decrease in the number of New Cytotoxic T-cells. There was an increased proportion of Cytotoxic thymocytes in ZD compared to MZD and CTL, which suggests that there might be fewer Cytotoxic T-cells exiting the thymus to enter the periphery. Using CD45RC and RT6.1 cell surface labeling, we have been able to characterize the maturation of splenocytes in ZD and found that the cell proportions during the first two stages of post-thymic maturation are not different

among dietary treatment groups, however, ZD rats have fewer late thymic emigrants ($TCR\alpha\beta^{+}CD90^{+}CD45RC^{+}RT6.1^{+}$) compared to CTL (18). Together, these results indicate that thymic export is maintained in ZD; however, the export of Helper T-cells is favored slightly over Cytotoxic T-cells and a mechanism other than apoptosis must be responsible for the lower proportion of late thymic emigrants in the spleens of ZD rats.

The explanation for the lower proportion of New T-cells, and more specifically late thymic emigrants, in dietary zinc deficiency is not evident. One possibility is that New T-cells are less able to proliferate in the zinc deficient state, resulting in a lower proportion of cells. In the present study we used DAPI to stain DNA and generate cell cycle histograms. Although the main focus of the present study was to determine differences among the dietary treatment groups in the proportion of lymphocyte subsets undergoing apoptosis, cell cycle histograms can also be used to determine the proportion of each cell type in the G2 phase of the cell cycle where dividing cells are located. There was no difference among the dietary groups in the proportion of any splenocyte subset in G2 of the cell cycle (data not shown) therefore, under these experimental conditions a reduced proliferative ability of New T-cells from ZD rats can not explain the differences seen. However, the cells in the present experiment were not exposed to a mitogenic stimulus that would promote proliferation, so this should not be conclusively ruled out as a possible explanation. CD90 is not only a maturational marker, but it is involved in T-cell activation (19). Perhaps in zinc

deficiency CD90 expression is down regulated in order to limit mitosis and preserve resources.

Dietary treatment did not affect the susceptibility to dexamethasone-induced apoptosis as evidenced by the lack of interaction between diet and treatment for the proportion of apoptotic thymocytes and splenocytes (Figure 8.7.5 and 8.7.6). However, there are some confounding results in this study. The proportion of Cytotoxic thymocytes was higher after DEX treatment, therefore, one would expect that Cytotoxic thymocytes are less susceptible to apoptosis (Table 8.6.2). However, the Cytotoxic thymocytes also represent the largest proportion of thymocytes in the apoptotic region after incubation and treatment with dexamethasone which would suggest that the Cytotoxic thymocytes are more susceptible to apoptosis (Figure 8.7.5). Dolzhanskiy and Basch (20) have reported that dexamethasone treatment in mouse thymocytes results in an increased proportion of Cytotoxic thymocytes and Pro-T cells in the surviving cells, with an increased proportion of Helper thymocytes in the apoptotic region. Their studies showed that during apoptosis, the Pre-T cells had reduced intensity levels of both CD4 and CD8 staining. The researchers speculated that the Pre-T cells were more susceptible to apoptosis and a portion of them lost expression of CD8 making them appear as Helper thymocytes thereby explaining the increased proportion of Helper thymocytes in the apoptotic region. In the present experiment, Pre-T cell proportions were maintained throughout the *in vitro* treatments, however, they represent the highest proportion of cells in G2 (7.6±0.3 vs approximately 1.7±0.1 for the other thymocyte

phenotypes). Perhaps in rats the Pre-T cells are able to multiply during the *in vitro* treatments, but some of them lose their CD4 cell surface expression. This would make the Pre-T cells appear as though they are Cytotoxic, which would explain the increase in Cytotoxic T-cells in the total population and in the apoptotic region.

In the spleen there was a similar conundrum: New T-cells represent a larger proportion of the splenocyte population with DEX treatment, and they also represent a larger proportion of the apoptotic cells. Perhaps in this situation, CD90 is up regulated during apoptosis, however, there are no published studies to support this hypothesis.

The MZD group has a similar body weight and feed intake as CTL; however, MZD along with ZD and DR had a higher proportion of splenic T-cells compared to CTL. In theory an increased proportion of T-cells should be a benefit to the organism in regards to fighting off infection; however, the function of the immune cells was not assessed in the present experiment. In studies of dietary zinc deficiency inclusion of a pair-fed group is essential in order to separate out the effects of malnutrition from zinc. The MZD group, which has reduced zinc status without the associated anorexia is also a valuable experimental group, because along with the DR group, it allows the researcher to separate the effects of ZD from malnutrition.

The present study indicates that immunodeficiency in the growing rat is not due to increased apoptosis of T-cells or their subsets. CD90 is involved in T-cell activation and the lower proportion of lymphocytes expressing this receptor in

ZD compared to CTL might explain the reduced ability for ZD rats to fight off infection. Future studies should examine at the cellular level why CD90 cell surface expression is down-regulated in dietary zinc deficiency and the down stream signaling effects of this change.

8.9 Literature Cited

1. Guilbert JJ. The world health report 2002 - reducing risks, promoting healthy life. Educ Health (Abingdon). 2003 Jul;16(2):230.

2. Umeta M, West CE, Haidar J, Deurenberg P, Hautvast JG. Zinc supplementation and stunted infants in ethiopia: A randomised controlled trial. Lancet. 2000 Jun 10;355(9220):2021-6.

3. Ninh NX, Thissen JP, Collette L, Gerard G, Khoi HH, Ketelslegers JM. Zinc supplementation increases growth and circulating insulin-like growth factor I (IGF-I) in growth-retarded vietnamese children. Am J Clin Nutr. 1996 Apr;63(4):514-9.

4. Shankar AH, Genton B, Baisor M, Paino J, Tamja S, Adiguma T, Wu L, Rare L, Bannon D, et al. The influence of zinc supplementation on morbidity due to plasmodium falciparum: A randomized trial in preschool children in papua new guinea. Am J Trop Med Hyg. 2000 Jun;62(6):663-9.

5. Sazawal S, Black RE, Jalla S, Mazumdar S, Sinha A, Bhan MK. Zinc supplementation reduces the incidence of acute lower respiratory infections in infants and preschool children: A double-blind, controlled trial. Pediatrics. 1998 Jul;102(1 Pt 1):1-5.

6. Shi HN, Scott ME, Stevenson MM, Koski KG. Energy restriction and zinc deficiency impair the functions of murine T cells and antigen-presenting cells during gastrointestinal nematode infection. J Nutr. 1998 Jan;128(1):20-7.

7. King LE, Osati-Ashtiani F, Fraker PJ. Apoptosis plays a distinct role in the loss of precursor lymphocytes during zinc deficiency in mice. J Nutr. 2002 May;132(5):974-9.

8. Lepage LM, Giesbrecht JA, Taylor CG. Expression of T lymphocyte p56(lck), a zinc-finger signal transduction protein, is elevated by dietary zinc deficiency and diet restriction in mice. J Nutr. 1999 Mar;129(3):620-7.

9. Moore JB, Blanchard RK, McCormack WT, Cousins RJ. cDNA array analysis identifies thymic LCK as upregulated in moderate murine zinc deficiency before T-lymphocyte population changes. J Nutr. 2001 Dec;131(12):3189-96.

10. Molina TJ, Kishihara K, Siderovski DP, van Ewijk W, Narendran A, Timms E, Wakeham A, Paige CJ, Hartmann KU, Veillette A. Profound block in thymocyte development in mice lacking p56lck. Nature. 1992 May 14;357(6374):161-4.

11. Levin SD, Anderson SJ, Forbush KA, Perlmutter RM. A dominant-negative transgene defines a role for p56lck in thymopoiesis. EMBO J. 1993 Apr;12(4):1671-80.

12. Di Somma MM, Nuti S, Telford JL, Baldari CT. p56lck plays a key role in transducing apoptotic signals in T cells. FEBS Lett. 1995 Apr 17;363(1-2):101-4.

13. Hosea HJ, Rector ES, Taylor CG. Zinc-deficient rats have fewer recent thymic emigrant (CD90+) T lymphocytes in spleen and blood. J Nutr. 2003 Dec;133(12):4239-42.

14. Hosseinzadeh H, Goldschneider I. Recent thymic emigrants in the rat express a unique antigenic phenotype and undergo post-thymic maturation in peripheral lymphoid tissues. J Immunol. 1993 Mar 1;150(5):1670-9.

15. Iwakoshi NN, Goldschneider I, Tausche F, Mordes JP, Rossini AA, Greiner DL. High frequency apoptosis of recent thymic emigrants in the liver of lymphopenic diabetes-prone BioBreeding rats. J Immunol. 1998 Jun 15;160(12):5838-50.

16. Jung CG, Kamiyama T, Agui T. Elevated apoptosis of peripheral T lymphocytes in diabetic BB rats. Immunology. 1999 Dec;98(4):590-4.

17. Fraker PJ, King LE, Lill-Elghanian D, Telford WG. Quantification of apoptotic events in pure and heterogeneous populations of cells using the flow cytometer. Methods Cell Biol. 1995;46:57-76.

18. Hosea HJ, Rector ES, Taylor CG. In: The lower proportion of CD90+ T-cells in zinc-deficient rats is due to a smaller proportion of late thymic emigrants. CSCN 5th annual scientific meeting: Healthy aging, inflammation in acute & chronic diseases; May 25-27; Edmonton, Alberta, Canada. ; 2006. p. CSCN abstract 25.

19. Loertscher R, Lavery P. The role of glycosyl phosphatidyl inositol (GPI)-anchored cell surface proteins in T-cell activation. Transpl Immunol. 2002 May;9(2-4):93-6.

20. Dolzhanskiy A, Basch RS. Flow cytometric determination of apoptosis in heterogeneous cell populations. J Immunol Methods. 1995 Mar 13;180(1):131-40.

9. ALTERED CYTOKINE PRODUCTION IN ZINC-DEFICIENT, DIET-RESTRICTED, AND MARGINALLY ZINC-DEFICIENT GROWING RATS IS NOT ASSOCIATED WITH SERUM CORTICOSTERONE CONCENTRATIONS

Heather J. Hosea[1], Edward S. Rector[2], and Carla G. Taylor[1]

Departments of Human Nutritional Sciences[1] and Immunology[2]

9.1 Abstract

The objective of the present study was to determine potential differences in the *in vitro* cytokine production [interleukin-2 (IL-2), interferon-γ (IFN-γ), IL-6 and IL-10] of isolated thymocytes and splenocytes after mitogenic stimulation with concavalin A (ConA). Weanling rats were assigned to one of four dietary treatments for 3 weeks: zinc-deficient (ZD, <1 mg zinc/kg diet, *ad libitum*), diet-restricted (DR, 30 mg zinc/kg diet, limited to the amount of feed as consumed by the ZD rats), marginally zinc-deficient (MZD, 10 mg zinc/kg diet, *ad libitum*) and control (CTL, 30 mg zinc/kg diet, *ad libitum*). At the end of the feeding trial, thymocytes and splenocytes were isolated for cytokine stimulation and determination of absolute numbers of T-cell phenotypes using flow cytometry and Flow-Count™ Fluorospheres. Serum corticosterone concentrations were determined by ELISA. ZD and DR had higher serum corticosterone concentrations compared to MZD and CTL. Thymocyte numbers (relative to thymus weight) were not altered by ZD, DR, or MZD, however, thymocyte IL-2 and IL-6 production in these groups was lower compared to CTL. ZD had a lower number of New Helper T-cells ($TCR\alpha\beta^+CD90^+4^+8^-$) and New Cytotoxic T-cells ($TCR\alpha\beta^+CD90^+4^-8^+$) as well as a lower production of Th1 cytokines (IL-2, IFN-γ) by stimulated splenocytes compared to DR, MZD and CTL. Splenocyte production of Th2 cytokines (IL-6, IL-10) was not affected by dietary treatment. These results indicate that zinc status, and not serum corticosterone concentration, is associated with altered cytokine production in zinc deficiency.

9.2 Introduction

The World Health Organization (WHO) has identified zinc deficiency as one of the leading risk factors of disease, ranking 5th in developing countries and 11th in developed countries (1). The 2002 WHO report estimates that zinc deficiency accounts for 18 percent of malaria cases, 16 percent of lower respiratory tract infections, and 10 percent of diarrhoeal disease worldwide. Infants and young children are among the population groups at most risk of zinc deficiency due to their increased requirements during growth (2).

Prasad (3) has reported that peripheral lymphocytes from zinc-deficient humans produce less interleukin (IL)-2 and interferon (IFN)-γ (often referred to as Th1 cytokines) while the Th2 cytokines (IL-6 and IL-10) were not affected. Th1 cytokines initiate a cellular response involving Natural Killer cells and cytotoxic T-cells which directly combat intracellular infections, but overproduction can lead to autoimmune diseases (4). Th2 cytokines stimulate the humoral response which is more effective at defending against intercellular infections, but overproduction of Th2 cytokines can lead to allergies (4). The balance between Th1 and Th2 cells is controlled mainly by the cytokines they produce. IFN-γ inhibits the cytokine production by Th2 cells, and IL-10 inhibits proliferation of Th1 cells (4).

Zinc has been shown to be essential for the expression of IL-2 receptors on lymphocytes (5). Sprietsma (6) has hypothesized that with fewer IL-2 receptors, Th1 cells cannot respond to nor produce the IL-2 and IFN-γ they need to proliferate, therefore, theTh2 producing cells and their cytokines begin to dominate. A shift in cytokine production from predominantly Th1 to

predominantly Th2 has been associated with a worsening clinical outcome in infections such as leprosy, tuberculosis and leishmaniasis (7). The decreased production of Th1 cytokines in dietary zinc deficiency might therefore, contribute to the increased infection risk in zinc deficiency.

Cytokines also play a role in thymocyte maturation (8); however, the effect of dietary zinc deficiency on thymocyte cytokine production has not been investigated. Theoretically, low IL-2 and IFN-γ concentrations in the thymus would limit T-cell proliferation and differentiation, resulting in reduced thymocyte numbers (thymic atrophy) and consequently fewer new T-cells being released into the periphery, thereby leading to lymphopenia and increased susceptibility to disease (8).

Peripheral IL-6 production (a Th2 cytokine) can stimulate the hypothalamus-pituitary-adrenal (HPA) axis leading to increased production of cortiocosterone by the adrenal gland (9). Corticosterone has been shown to suppress production of IFN-γ and IL-2 by splenic Th1 cells thereby pushing the cytokine balance towards Th2 (10). Thus, we hypothesized that the low zinc status during dietary zinc deficiency shifts cytokine production in favor of a Th2 response and this shift in cytokine production is compounded by the higher serum corticosterone concentrations associated with dietary zinc deficiency.

The objective of the present study was to determine potential differences in the *in vitro* cytokine production (IL-2, IFN-γ, IL-6 and IL-10) of isolated thymocytes and splenocytes after mitogenic stimulation with concavalin A (ConA) in zinc-deficient, diet-restricted, marginally zinc-deficient and control growing rats.

Serum corticosterone concentrations were also assessed to explore the potential association between changes in cytokine production and stress hormone levels. Flow cytometric analysis was used to enumerate thymic and splenic T-cell subsets using Flow-Count™ Fluorospheres.

9.3 Materials and Methods

9.3.1 Animals and diets

Weanling Sprague Dawley rats (Charles River Laboratories, St. Constant, PQ) were acclimatized for 5 days and randomly assigned to one of four dietary treatment groups for three weeks: zinc-deficient (ZD; <1 mg zinc/kg), marginally zinc-deficient (MZD; 10 mg zinc/kg), nutritionally complete control diet (CTL; 30 mg zinc/kg), and diet-restricted (DR; fed the control diet, but only the amount of feed as consumed on the previous day by the individual zinc deficient rat paired to the DR rat). The diet-restricted group controls for the effects of undernutrition commonly seen in zinc deficiency. Body weight was measured weekly and feed intake was measured daily.

The experimental diets (based on the AIN-93G formulation and previously described by Lepage (11)) containing egg white, additional biotin (2 mg/kg diet) and potassium phosphate were fed *ad libitum*, except for the diet-restricted group, and distilled water was provided. Zinc content of the diets was verified by atomic absorption analysis. To avoid zinc recycling and contamination, the rats were housed individually in stainless steal hanging cages with mesh bottoms so urine and feces could drop to the shavings below. In addition, the zinc-deficient

group was placed on the upper rows of the cage rack. The rats were maintained in an environment of controlled temperature (21-23°C), humidity (55%) and light cycle (14 hours light/10 hours dark). Animal care was provided in accordance with a protocol approved by the University of Manitoba Protocol Management and Review Committee.

9.3.2 Sample collection

At the end of the feeding trial, the rats were euthanized by CO_2 asphyxiation and decapitation. Trunk blood was collected and centrifuged (Beckman TJ-6R Tabletop centrifuge) to obtain serum and stored at -80°C until analysis. The spleen and thymus were removed aseptically, weighed and then processed immediately. Femurs were removed, cleaned of soft tissue and stored at -20°C until analysis.

Cell Preparation

Single cell suspensions of spleen and thymus were prepared by pressing tissues through nylon screens into Hank's buffered saline supplemented with 10 mM HEPES, 4% dextran charcoal absorbed fetal bovine serum (Hyclone, Logan, UT), and 1% antibiotic/antimycotic at pH 7.4. Dextran charcoal absorbed fetal bovine serum was used to limit the amount of external steroids the splenocytes were exposed to during cell preparation. Red blood cells in the spleen cell suspensions were lysed using ammonium chloride lysis buffer (155 mM NH_4Cl, 0.1 mM EDTA, and 10 mM $KHCO_3$ at pH 7.2). Cell concentration and viability were determined using trypan blue dye exclusion on an AO Bright-Line Hemacytometer (American Optical Corporation, Buffalo, NY).

Cytokine Stimulation

Thymocytes and splenocytes (3 x 10^6 cells) from each rat were resuspended in 3 mL sterile RPMI-1640 supplemented with 10 mmol/L HEPES, 10 mmol/L sodium bicarbonate, 1 mmol/L soidium pyruvate, 2 mmol/L glutamine, 0.1 mmol/L non-essential amino acids, 50 µmol/L 2-mercaptoethanol, 1% antibiotic/antimycotic and 5% fetal bovine serum at pH 7.2. ConA (300 µL of 2.5 µg/mL working solution) was added and cells were incubated at 37°C with 5% CO_2 for 48 h. Cells not exposed to mitogen were included as a negative control to verify that mitogenic stimulation was effective in increasing cytokine production. After the 48 h incubation, cells were centrifuged for 5 minutes at 300 g. Cell culture supernatants were collected and stored at -80°C until analysis.

9.3.3 Outcome measurements

Zinc Analysis

After obtaining wet and dry weights, femurs and diet samples were wet-ashed using trace element grade nitric acid. After appropriate dilution of digests or serum, zinc concentration was determined by atomic absorption spectrometry (Varian Spectra AA, Varian Australia, Mulgrave, VI). Bovine liver standard reference material 1577b (US Department of Commerce, National Institute of Standards and Technology, Gaithersburg, MD) was used as the quality control.

Determination of T-lymphocyte Subpopulations

Antibodies: Monoclonal antibodies for TCRαβ (PE label, clone R73, isotype mouse $IgG_{1,k}$), CD90 (FITC label, Thy1.1, clone OX-7, isotype mouse $IgG_{1,k}$), CD4 (PE-Cy5 label, clone OX-35, isotype mouse $IgG_{2a,k}$), and CD8 (biotin, clone

OX-8, isotype mouse $IgG_{1,k}$) were obtained from BD Pharmingen (Mississauga, ON). Streptavidin-PE-Cy7 conjugate (BD Pharmingen) was used to detect the biotinylated antibodies.

Cell Labeling and Flow Cytometry: Cells from each dietary treatment group and tissue were washed and placed in label buffer (phosphate buffered saline containing 23 mM sodium azide and 2% fetal bovine serum, pH 7.4) at 4°C at a concentration of 1×10^6 cells/mL. The biotinylated anti-CD8 antibody was added first, incubated for 30 minutes at 4°C, followed by a wash step. Then, the directly conjugated antibodies and streptavidin-PE-Cy7 were added, incubated as previously described, followed by a wash step. Samples were resuspended in phosphate buffered saline containing 50% fetal bovine serum. Ethanol (70%) was added drop wise while gently mixing to a final concentration of 50%. Cells were stored overnight at 4°C and analyzed on the flow cytometer the following morning.

Flow cytometry analysis was performed on a Beckman Coulter EPICS ALTRA (Beckman Coulter, Mississauga, ON) high-speed cell sorter with laser excitation tuned to 488 nm (65 mW) and UV. Forward versus side scatter histograms were used to gate on lymphoid cells. The fluorescence signals were separated with the standard dichotic long pass filters provided with the instrument and detected through 525 nm (FITC), 575 nm (PE), 675 nm (PE-Cy5) and 735 nm (PE-Cy7) bandpass filters. The data was collected in listmode format with the subsequent analyses based on 20 000 cells satisfying the light scatter gate and $CD90^+$ in thymocytes (Figure 9.7.1), or 20 000 cells gated on

light scatter, TCRαβ$^+$, and CD90$^-$ in splenocytes (Figure 9.7.2) using the EXPO32 MultiCOMP MFA software provided with the instrument. Flurochrome-isotype matched controls were prepared to assess autofluorescence and nonspecific binding, while single color samples were employed to adjust color compensation. T-cell subset numbers were calculated using Flow-Count™ Fluorospheres (Beckman Coulter, Mississauga, ON) based on the total number of cells counted, the total number of fluorospheres counted and the original concentration of the fluorospheres. Cell counts were corrected by the appropriate dilution factor.

Cytokine analysis

IL-2, IL-6, IL-10, and IFN-γ in cell culture supernatants were determined simultaneously using a cytometric bead array kit (LINCO Research, St. Charles, MO). The standard curves were 4.88-20 000 pg/mL. The samples were analyzed using the Luminex™ 100 instrument (Luminex Corporation, Austin, TX).

Corticosterone concentration

Serum corticosterone concentrations were determined using an enzyme immunoassay kit (Assay Designs, Ann Arbor, MI).

9.4 Statistical Analyses

Data was analyzed by one-way ANOVA using the general linear models procedure or repeated measure ANOVA (SAS software release 9.1, SAS Institute, Cary NC) as appropriate. Significant differences among means were determined using Duncan's multiple range test. Differences were considered significant at $p<0.05$.

9.5 Results

Feed intake. After only three days of treatment, the ZD group consumed 17% less feed per day than CTL and MZD (Figure 9.7.3). By day four, ZD was consuming 54% less feed/day than MZD and CTL and this continued until the end of the study. The MZD group did not exhibit any signs of anorexia throughout the study.

Body weight. The MZD group had the same body weight (Figure 9.7.4), weight gain and feed efficiency ratio (Table 9.6.1) as CTL throughout the study. The ZD group weighed 22%, 40%, and 50% less than CTL at weeks 1, 2, and 3, respectively. Of the 35.8 g of total weight gained by ZD, 25.5 g was gained during the first week and only 10.3 g was gained during the remaining 2 weeks, while the MZD and CTL groups gained 61.0 g in the first week, 56.5 g in the 2nd week and 54.2 g in the 3rd week. DR weighed more than ZD at each week due to more efficient conversion of feed into growth as indicated by a lower feed efficiency ratio.

Serum and femur zinc. Femur and serum zinc concentrations were reduced 78-83% in ZD and 38-40% in MZD rats compared to CTL (Table 9.6.1). Also, serum and femur zinc concentrations were 15% lower in DR compared to CTL.

Lymphoid organ weight. One of the classic characteristics of ZD is reduced lymphoid organ weight. The thymus and spleen weights of ZD and DR were not significantly different from each other, but they were 45% and 48% lower, respectively, than MZD and CTL (Tables 9.6.2 and 9.6.3). When the thymus and

spleen weights were corrected for body weight, there were no differences among the dietary treatments.

Thymocyte subset numbers. ZD had 39% fewer Pre-T cells ($90^+4^-8^-$), 57% fewer Pro-T cells ($90^+4^+8^+$), and 51% fewer Helper T-cells ($90^+4^+8^-$) per thymus, respectively, compared to MZD and CTL, but not DR (Table 9.6.2). However, there was no difference among dietary treatment groups in the number of Cytotoxic T-cells ($90^+4^-8^+$) per thymus. Once the thymocyte numbers were corrected for thymus weight, there were no differences among the dietary treatment groups in the number of thymocyte subsets (Pre, Pro, Helper and Cytotoxic) per gram of thymus.

Splenocyte T-cell subset numbers. Both cytotoxic ($CD8^+$) and helper ($CD4^+$) lymphocytes contributed to the changes in the total new ($CD90^+$) and mature ($CD90^-$) T-cell populations among the dietary treatment groups. ZD had 41% fewer New Cytotoxic T-cells ($TCR\alpha\beta^+90^+4^-8^+$) and 42% fewer New Helper T-cells ($TCR\alpha\beta^+90^+4^+8^-$) per spleen compared to DR, while DR had 53% fewer New Cytotoxic T-cells and 51% fewer New Helper T-cells per spleen compared to MZD and CTL (Table 9.6.3). ZD also had 65% fewer Mature Cytotoxic T-cells ($TCR\alpha\beta^+90^-4^-8^+$) and 56% fewer Mature Helper T-cells ($TCR\alpha\beta^+90^-4^+8^-$) per spleen compared to MZD and CTL, while DR had 56% fewer Mature Cytotoxic T-cells per spleen compared to MZD and 46% fewer Mature Helper T-cells per spleen compared to MZD and CTL. When the cell numbers were corrected for spleen weight, ZD had 44% fewer New Cytotoxic T-cells and 43% fewer New Helper T-cells per gram of spleen compared to DR, MZD and CTL, but there was

no difference among the dietary treatment groups in the number of Mature Helper or Mature Cytotoxic T-cells.

Serum corticosterone concentration. ZD and DR had a 14-fold higher serum corticosterone concentrations compared to MZD and CTL (Figure 9.7.5).

Cytokine concentrations after mitogenic stimulation. Mitogenic stimulation of thymocytes from ZD, DR and MZD resulted in 40-42% lower concentrations of IL-2 and IL-6 in cell culture supernatants compared to CTL (Figure 9.7.6). There were no differences among the dietary treatment groups in the concentration of IFN-γ and IL-10 produced by thymocytes after stimulation.

ZD splenocytes produced 54% less IL-2 compared to DR, MZD and CTL, and 71% less IFN-γ compared to DR and MZD in response to ConA. IL-6 and IL-10 concentrations were not different among dietary treatment groups, and IL-10 concentrations from stimulated splenocytes were not different from un-stimulated splenocytes.

9.6 Tables

Table 9.6.1. Weight gain, feed efficiency ratio and zinc status of zinc-deficient, marginally zinc-deficient, diet-restricted, and control rats[1].

	Dietary Group[2]			
	ZD	DR	MZD	CTL
Total weight gain (g)	35.8 ± 1.7[c]	54.9 ± 3.9[b]	170.1 ± 3.3[a]	173.3 ± 4.7[a]
Feed efficiency ratio [(3)]	6.1 ± 0.3[a]	4.2 ± 0.2[b]	2.4 ± 0.1[c]	2.4 ± 0.1[c]
Serum zinc (µmol/L)	5.2 ± 0.3[d]	26.2 ± 1.2[b]	18.5 ± 0.9[c]	30.6 ± 1.3[a]
Femur zinc (µmol/g dry bone)	1.0 ± 0.1[d]	4.1 ± 0.1[b]	3.0 ± 0.1[c]	4.8 ± 0.1[a]

[1]Values are means ± SEM, n=8. Different superscript letters indicate significant differences among means, $p < 0.05$.

[2]ZD=zinc-deficient group; DR=diet-restricted group; MZD=marginally zinc-deficient group; CTL=control group.

[3]Feed efficiency ratio = total feed intake (g)/total weight gain (g).

Table 9.6.2. Thymus weight and thymocyte subset numbers of zinc-deficient, marginally zinc-deficient, diet-restricted, and control rats[1,2].

	Dietary Group[3]			
	ZD	DR	MZD	CTL
Thymus weight (mg)	418 ± 18.3[b]	528 ± 20.6[b]	882 ± 35.3[a]	828 ± 72.6[a]
Total thymocytes (#/thymus*10^9)	51.5 ± 6.1[b]	73.7 ± 9.8[ab]	111±16.3[a(4)]	104 ± 16.7[a]
Pro-T cell	1.8 ± 0.1[b]	2.4 ± 0.3[ab]	3.0 ± 0.3[a(5)]	2.9 ± 0.4[a]
Pre-T cell	40.2 ± 5.7[c]	59.0 ± 8.2[bc]	104 ± 18.8[a]	83.1 ±14.4[ab]
Cytotoxic	4.4 ± 0.7	4.7 ± 0.5	6.4 ± 0.7[(6)]	6.2 ± 0.9
Helper	5.1 ± 0.6[b]	7.6 ± 1.2[ab]	11.0 ±1.3[a(7)]	9.9 ±1.2[a(8)]
Thymus(mg)/bwt(g)	3.0 ± 0.1	3.2 ± 0.1	3.2 ± 0.1	3.0 ± 0.2
Total thymocytes (#/g thymus*10^9)	123 ± 13.5	138 ± 16.3	142 ± 21.2	127 ± 17.3
Pro-T cell	4.3 ± 0.3	4.5 ± 0.6	4.0 ± 0.6	3.6 ± 0.4
Pre-T cell	95.9 ± 12.5	111 ± 14.1	115 ± 18.5	101 ± 14.8
Cytotoxic	9.3 ± 1.4[(9)]	8.9 ± 0.7	8.6 ± 1.4	7.3 ± 0.8
Helper	12.4 ± 1.4	14.2 ± 2.1	14.4 ± 2.2	14.8 ± 2.5

[1]Values are means ± SEM, n=8, except as indicated in footnotes 4-9. Different superscript letters indicate significant differences among means, $p < 0.05$.

[2]Total thymocytes identified as $CD90^+$; Pre-T cells identified as $CD90^+4^-8^-$; Pro-T cells identified as $CD90^+4^+8^+$; Cytotoxic identified as $CD90^+4^-8^+$; and Helper identified as $CD90^+4^+8^-$ by flow cytometry.

[3]ZD=zinc-deficient group; DR=diet-restricted group; MZD=marginally zinc-deficient group; CTL=control group.

[4-9]Data point removed from analysis because it was greater than 3 standard deviations from the mean (value= 243, 7.44, 15.9, 24.6, 24.9, 21.5, respectively).

Table 9.6.3. Spleen weight and T-cell subset numbers of zinc-deficient, marginally zinc-deficient, diet-restricted, and control rats[1,2].

	Dietary Group[3]			
	ZD	DR	MZD	CTL
Spleen weight (mg)	377 ± 14.2[b]	402 ± 14.1[b]	744 ± 40.4[a]	747 ± 26.3[a]
Total T-cells (#/spleen*10^7)	447 ± 90.2[b]	613 ± 80.7[b]	1386 ± 263[a]	1061± 95.3[a]
Total New T-cells	41.1 ± 8.6[c]	71.7 ± 10.3[b]	163 ± 26.6[a]	128 ± 13.2[a]
New Cytotoxic	15.7 ± 3.8[c]	26.6 ± 4.1[b]	64.9 ± 11.9[a]	48.0 ± 5.8[a]
New Helper	20.3 ± 4.3[c]	35.4 ± 5.2[b]	79.8 ± 13.0[a]	63.1 ± 7.1[a]
Total Mature T-cells	396 ± 80.4[b]	528 ± 70.0[b]	1189 ± 235[a]	900 ± 85.7[a]
Mature Cytotoxic	144 ± 30.6[c]	213 ± 33.2[bc]	482 ± 97.7[a]	338 ± 38.9[ab]
Mature Helper	228 ± 47.5[b]	283 ± 33.4[b]	545±96.0[a(4)]	504 ± 51.3[a]
Spleen(mg)/bwt(g)	2.7 ± 0.1	2.5 ± 0.1	2.7 ± 0.1	2.7 ± 0.1
Total T-cells (#/g spleen*10^7)	1156 ± 205	1523 ± 201	1790 ± 269	1433 ± 141
Total New T-cells	106 ± 19.2[b]	178 ± 25.9[a]	213 ± 25.1[a]	173 ± 18.4[a]
New Cytotoxic	40.3 ± 8.6[b]	66.0 ± 10.4[a]	84.0 ± 11.6[a]	64.3 ± 7.8[a]
New Helper	52.8 ± 9.5[b]	87.7 ± 12.9[a]	104 ± 12.5[a]	84.9 ± 10.0[a]
Total Mature T-cells	1025 ± 183	1313 ± 174	1533 ± 245	1216 ± 127
Mature Cytotoxic	373 ± 70.1	530 ± 83.5	621 ± 102	456 ± 55.4
Mature Helper	590 ± 109	702 ± 82	722 ± 93[(5)]	680 ± 75.7

[1]Values are means ± SEM, n=8, except as indicated in footnotes 4-5. Different superscript letters indicate significant differences among means, $p < 0.05$.

[2]Total T-cells identified as $TCR\alpha\beta^{+}$; Total New T-cells identified as $TCR\alpha\beta^{+}CD90^{+}$; New Cytotoxic identified as $TCR\alpha\beta^{+}CD90^{+}4^{-}8^{+}$; New Helper identified as $TCR\alpha\beta^{+}CD90^{+}4^{+}8^{-}$; Total Mature T-cells identified as $TCR\alpha\beta^{+}CD90^{-}$; Mature Cytotoxic identified as $TCR\alpha\beta^{+}CD90^{-}4^{-}8^{+}$; and Mature Helper identified as $TCR\alpha\beta^{+}CD90^{-}4^{+}8^{-}$ by flow cytometry.

[3]ZD=zinc-deficient group; DR=diet-restricted group; MZD=marginally zinc-deficient group; CTL=control group.

[4-5]Data point removed from analysis because it was greater than 3 standard deviations from the mean (value= 1317, and 1571, respectively)

9.7 Figures

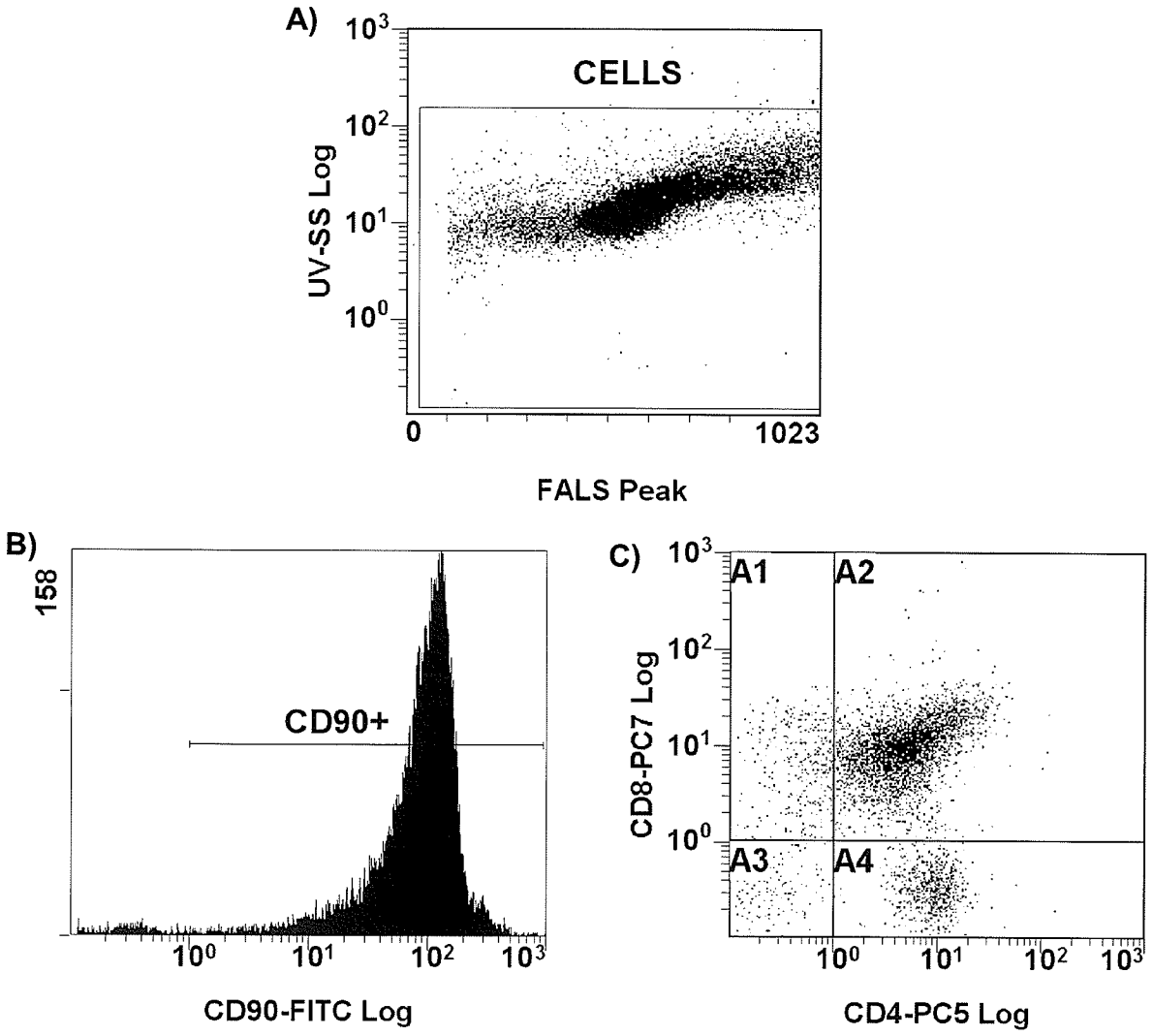


Figure 9.7.1. Representative flow cytometry plot of thymocytes. Definition of lymphocytes (CELLS) by forward vs side scatter [A]; Definition of CD90 binding after gating on CELLS [B]; CD8 and CD4 binding after gating on $CD90^+$ CELLS [C].

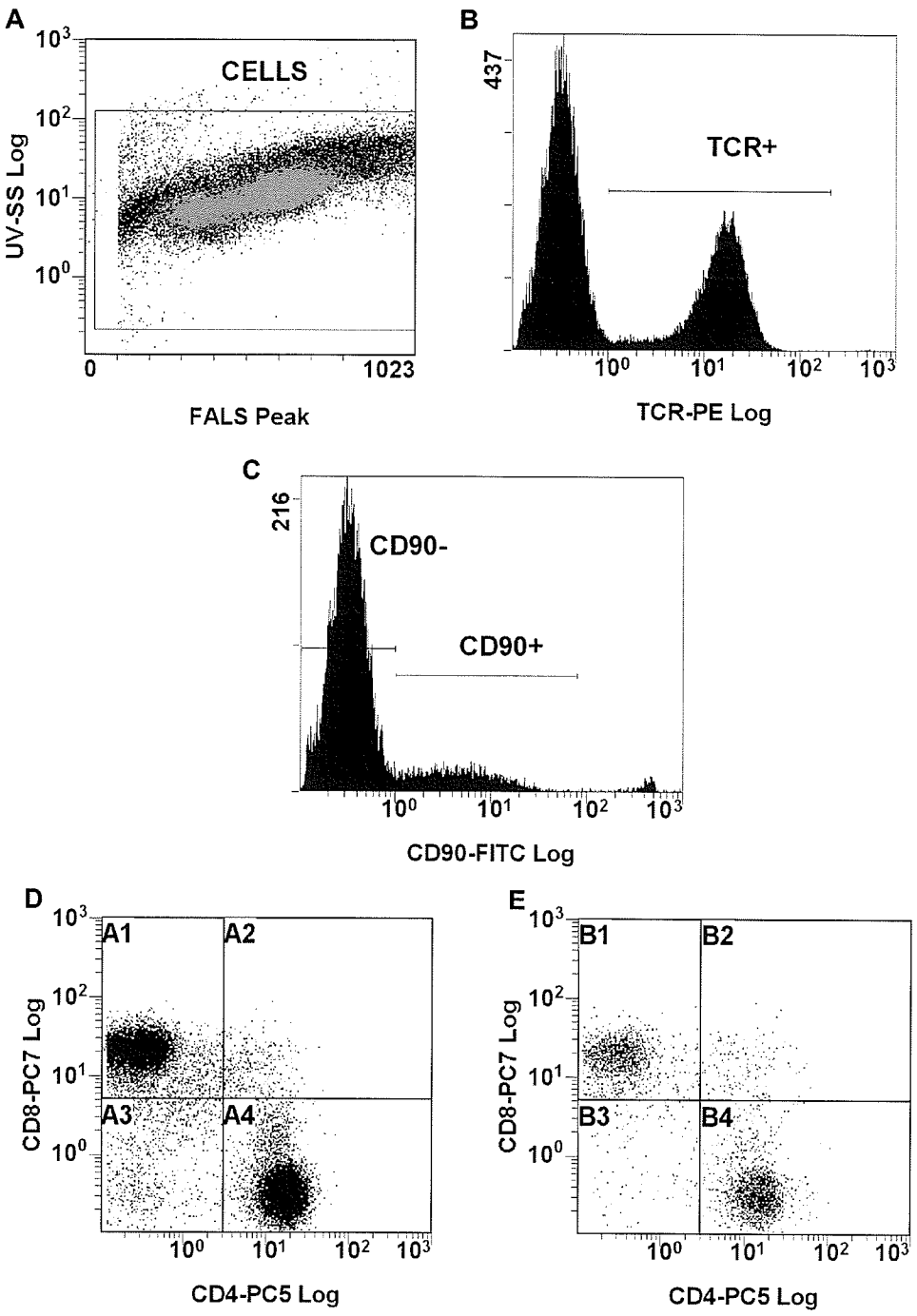


Figure 9.7.2. Representative flow cytometry plot of splenocytes. Definition of lymphocytes (CELLS) by forward vs side scatter [A]; Definition of TCRαβ binding after gating on CELLS [B]; Definition of CD90 binding after gating on $TCR\alpha\beta^+$ CELLS [C]; CD8 and CD4 binding after gating on $CD90^-$ $TCR\alpha\beta^+$ CELLS [D]; CD8 and CD4 binding after gating on $CD90^+$ $TCR\alpha\beta^+$ CELLS [E].

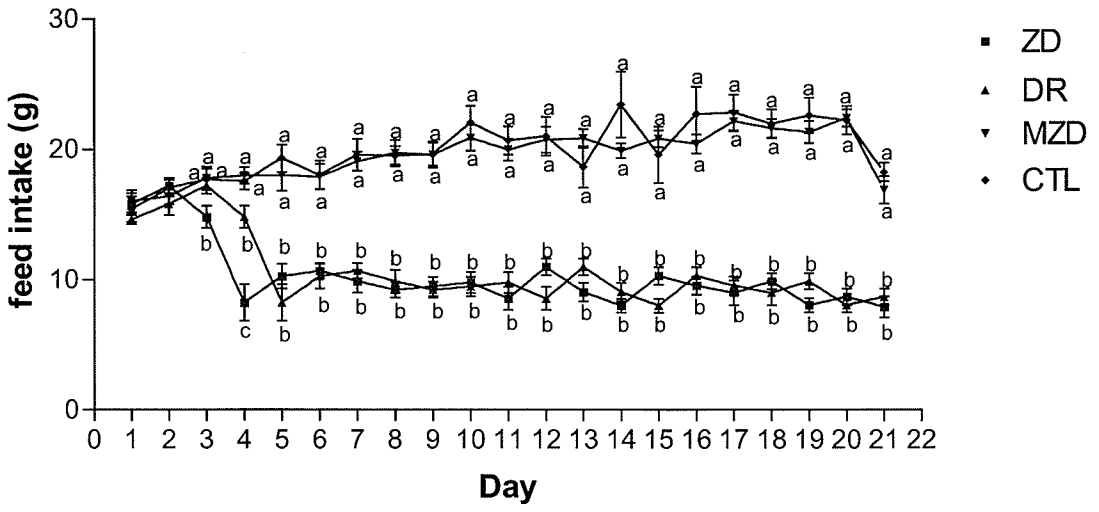


Figure 9.7.3. Feed intake of zinc-deficient, marginally zinc-deficient, diet-restricted and control rats over 21 days. Values are means ± SEM for n=8 rats. There were significant main effects of diet ($p<0.0001$), time ($p<0.0001$) and diet*time interaction ($p<0.0001$). At each time point, different letters indicate significant differences among means ($p<0.05$).

Abbreviations: ZD=zinc-deficient group; DR=diet-restricted group; MZD=marginally zinc-deficient group; CTL=control group.

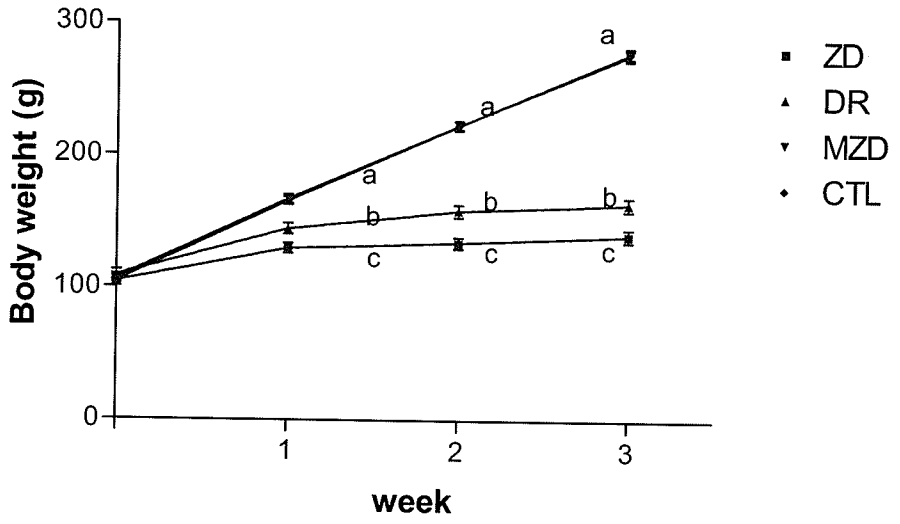


Figure 9.7.4. Weekly body weight of zinc-deficient, marginally zinc-deficient, diet-restricted and control rats. Values are means ± SEM for n=8 rats. There were significant main effects of diet ($p<0.0001$), time ($p<0.0001$) and diet*time interaction ($p<0.0001$). Different letters indicate significant differences among means ($p<0.05$). The values for MZD and CTL are overlapping. Abbreviations: ZD=zinc-deficient group; DR=diet-restricted group; MZD=marginally zinc-deficient group; CTL=control group.

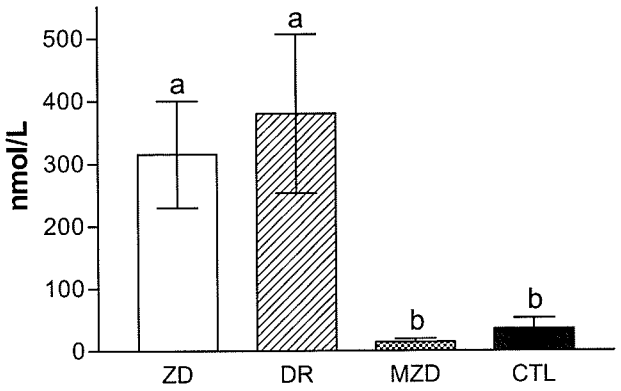
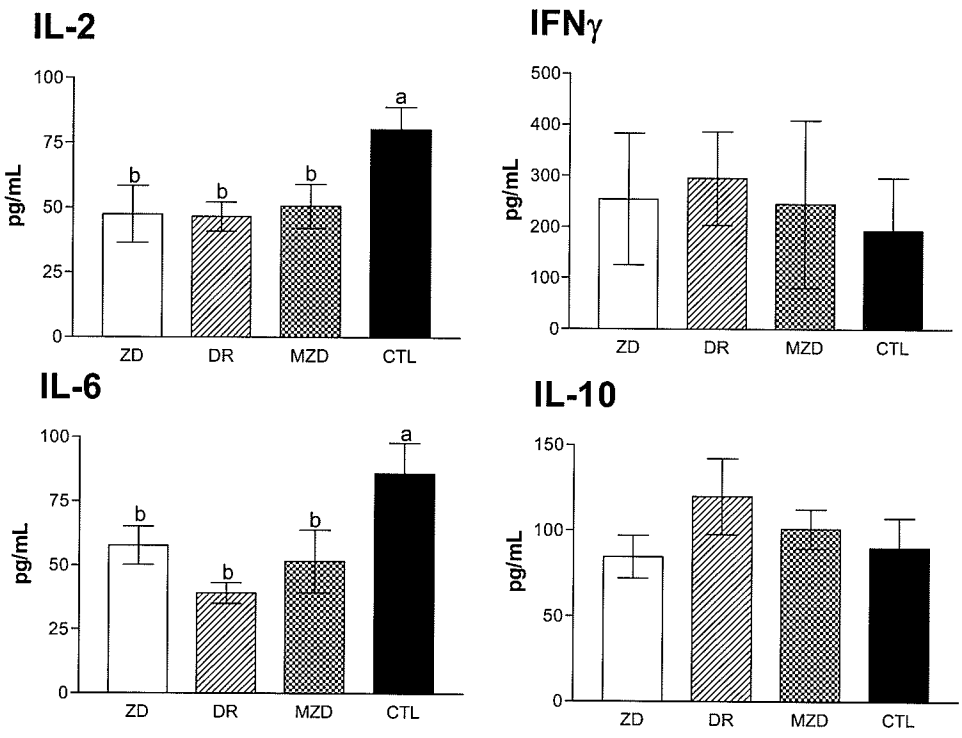


Figure 9.7.5. Serum corticosterone concentrations of zinc-deficient, diet-restricted, marginally zinc-deficient and control rats. Values are means ± SEM for n=8 rats. Different letters indicate significant differences among means ($p<0.05$).

Abbreviations: ZD=zinc-deficient group; DR=diet-restricted group; MZD=marginally zinc-deficient group; CTL=control group.

THYMUS



SPLEEN

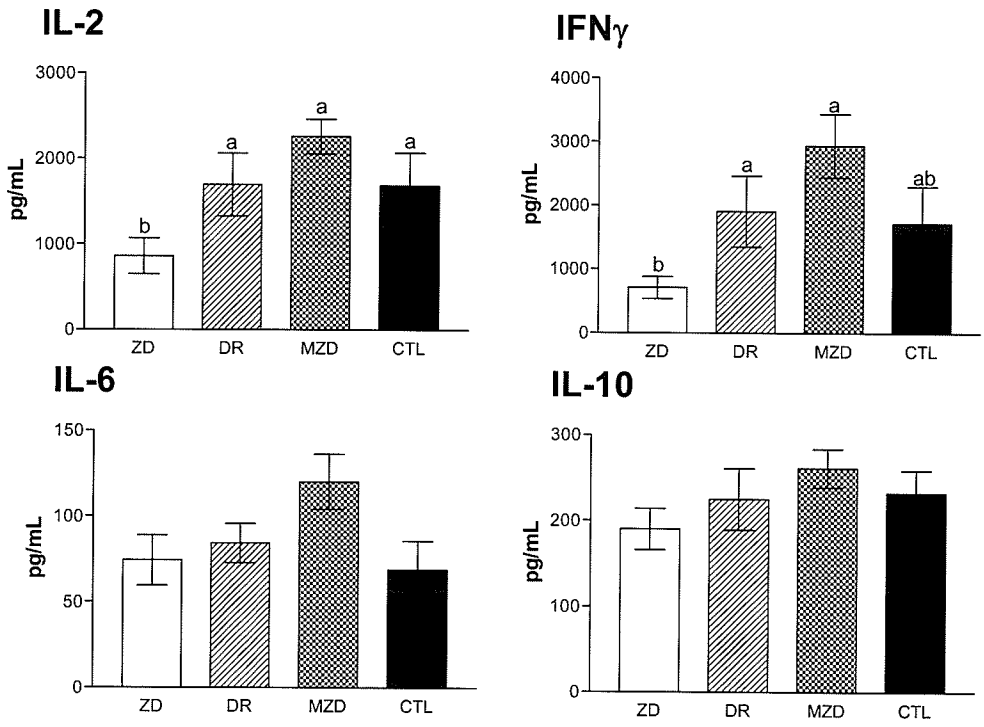


Figure 9.7.6. Cytokine concentrations of concanvalin A stimulated thymocytes and splenocytes from zinc-deficient, diet-restricted, marginally zinc-deficient and control rats. Thymocytes and splenocytes were stimulated in vitro with concanvalin A for 48 h and cytokine production was measured as described in the Methods. Values are means ± SEM for n=7 (thymocytes) or n=8 (splenocytes), with the following exceptions where data points were removed because they were greater than 3 standard deviations from the mean: n=6, DR thymocyte IL-2 (value removed=226.8) and n=7, DR splenocyte IL-6 (value removed=364.5). Different letters indicate significant differences among means ($p<0.05$). There were no differences among the dietary treatment groups in the un-stimulated cytokine concentrations. Un-stimulated thymocyte cytokine concentrations were as follows: IL-2= 7.0 ± 1.3 pg/mL; IL-6= 17.3 ± 4.2 pg/mL; IL-10= 19.8 ± 2.4 pg/mL; IFN-γ= 11.5 ± 3.0 pg/mL. Un-stimulated splenocyte cytokine concentrations were as follows: IL-2= 16.8 ± 1.7 pg/mL; IL-6= 45.2 ± 4.7 pg/mL; IL-10= 286.9 ± 14.3 pg/mL; IFN-γ= 5.5 ± 0.9 pg/mL. Abbreviations: ZD=zinc-deficient group; DR=diet-restricted group; MZD=marginally zinc-deficient group; CTL=control group.

9.8 Discussion

Researchers studying the effects of dietary zinc deficiency typically include a pair-fed group (identified as DR in the present study) to separate out the effects of energy malnutrition from zinc deficiency. Because severe zinc deficiency in humans is rare, a marginally zinc deficient group was also included in the present study. The MZD group had reduced zinc status compared to both CTL and DR; however, it did not exhibit any signs of anorexia or impaired growth compared to CTL (Table 9.6.1, Figure 9.7.3 and 9.7.4). The DR group had stunting malnutrition similar to the ZD group; however, their zinc status was greater than both ZD and MZD. Therefore, this study was able to examine the effects of reduced zinc status without malnutrition as well as being able to separate the effects of zinc status from energy malnutrition.

The major finding in the present study was that the altered cytokine production by thymocytes and splenocytes during dietary zinc deficiency, diet-restriction and marginal zinc deficiency could not be attributed to serum corticosterone concentrations. ZD and DR had higher serum corticosterone concentrations compared to MZD and CTL; however, ZD, DR and MZD had lower IL-2 and IL-6 cytokine production by ConA stimulated thymocytes (not reported previously) while only ZD had lower IL-2 cytokine production by ConA stimulated splenocytes compared to CTL. Due to the inclusion of the DR and MZD groups, the present study was able to separate out the effects of elevated corticosterone concentrations from the effects of zinc deficiency.

Thymocytes from ZD, DR and MZD groups produced similar cytokine profiles (lower concentrations of IL-2 and IL-6 compared to CTL)(Figure 9.7.5). In the

periphery, IL-2 and IL-6 are identified as Th1 and Th2 cytokines, respectively, so it would appear that in the thymus there is not a shift in Th1-Th2 balance. However, in the thymus, both IL-2 and IL-6 play a role in stimulating thymocyte proliferation and differentiation (8). Less IL-2 and IL-6 indicates that ZD, DR and MZD thymocytes are receiving fewer signals to proliferate and differentiate compared to CTL, suggesting that thymocyte numbers should be negatively affected. In fact, blocking the IL-2 receptor in rat fetal thymus organ cultures results in 21% fewer thymocytes (12) and IL-6 deficient mice have 20-40% fewer thymocytes and peripheral T-cells (13). On a per gram basis, ZD and DR were able to maintain thymocyte numbers (Table 9.6.2) despite their cytokine profile, most likely because they are not growing as quickly as MZD and CTL. However, growth of MZD was similar to CTL and yet MZD were able to maintain thymocyte numbers despite lower concentrations of IL-2 and IL-6 being produced. A possible explanation is that there is a redundancy in the function of these cytokines and other cytokines are able to maintain proliferation and differentiation when IL-2 and IL-6 concentrations are low (8).

Because ZD and DR have lower thymus weights (Table 9.6.2), it follows that they should have fewer thymocytes per thymus compared to MZD and CTL. Previous work using the adult mouse as a model reported no differences between ZD and CTL mice in the proportion of thymocyte subsets, but did not determine absolute numbers of each subset (14). In the present study, ZD had fewer Pre-, Pro-, and Helper thymocytes compared to MZD and CTL, but there was no difference in the number of Cytotoxic thymocytes among the dietary treatment groups. TCR signaling through $p56^{lck}$ promotes CD8 differentiation (15), and

thymocyte $p56^{lck}$ levels have been reported as higher in ZD compared to CTL (16) which might explain the higher than expected number of Cytotoxic thymocytes.

ZD rats had fewer New T-cells (both Helper and Cytotoxic) per spleen and per gram of spleen compared to DR, MZD, and CTL (Table 9.6.2). New T-cells are essential to ensure T-cell repertoire diversity (17), thus over time the ZD rat may become more vulnerable to infection. We have previously reported that ZD rats have a lower proportion of New T-cells ($TCR\alpha\beta^{+}CD90^{+}$), however, it was not clear whether the lower proportion was due to a lower number of New Helper T-cells, a lower number of New Cytotoxic T-cells or both (16). The present study builds on this previous work by reporting that there was a specific effect of severely reduced zinc status on the number of both New Helper T-cells and New Cytotoxic T-cells (Table 9.6.3).

Only ZD splenocytes produced less IL-2 compared to CTL indicating a specific effect of severe zinc deficiency on peripheral T-cell cytokine production. These results support previous findings in ZD humans (18) and provide a possible explanation for the lower number of new peripheral T-cells. New T-cells respond to cytokine proliferation signals from IL-2 without any other stimulus and this response is lost as T-cells develop in the periphery (19). This could explain why the New T-cells would be more vulnerable to low IL-2 concentrations than the Mature T-cells resulting in a lower number of the New T-cells but not the Mature T-cells. IFN-γ pushes Th1 cells to produce their cytokines (4). A lack of a difference in splenocyte IL-2 production among DR, MZD and CTL may be explained by the higher production of IFN-γ by the splenocytes in these groups compared to ZD. A report by

Shi and colleagues (20) indicated that splenic T-cells from ZD mice that were infected by parasite (Heligmosomoides polygyrus) produce less IL-4 and IL-5 (Th2 cyokines not examined in the present study) compared to CTL and DR when exposed to the parasite antigen *in vitro*, suggesting that Th2 cytokine production can also be lowered by ZD. Taken together, these studies suggest that there is not a preferential shift in cytokine production due to ZD, however, there is an overall decreased ability to respond to antigen in ZD and the shift in cytokine production may be reflecting whether the stimulus elicits a predominantly Th1 or Th2 response.

It is also interesting to note that IL-6 production was not affected by dietary treatment despite a higher serum corticosterone concentration in ZD and DR compared to MZD and CTL. IL-6 is known as a glucocorticoid increasing factor, which stimulates the hypothalamus-pituitary-adrenal axis to increase the release of corticotrophin releasing hormone in the paraventricular nucleus of the hypothalamus, which stimulates secretion of pituitary adrenaocorticotropic hormone (ACTH) which turns on the production of corticosterone in the adrenal gland (21). A previous study by our laboratory showed that plasma ACTH and serum TNF-α concentrations were not altered by ZD or DR compared to CTL (22), thus the signaling mechanisms for elevated serum corticosterone require further elucidation.

In summary, the present study indicates that severe zinc deficiency, but not serum corticosterone concentrations, were associated with a lower number of both New Cytotoxic T-cells and New Helper T-cells in spleen as well as lower production of Th1 cytokines (IL-2, IFN-γ) but not Th2 cytokines (IL-6, IL-10) by stimulated splenocytes. Cytokine production (IL-2 and IL-6) by stimulated thymocytes was

reduced in ZD, DR and MZD despite no alteration in thymocyte numbers relative to thymus weight. The results also indicate that a mild reduction in zinc status (MZD group) can alter cell function and that cell dysfunction can be detected before any phenotypic alterations occur. This study demonstrates the value of including a marginally zinc-deficient group, a model that may be more applicable to humans.

9.9 Literature Cited

1. Guilbert JJ. The world health report 2002 - reducing risks, promoting healthy life. Educ Health (Abingdon). 2003 Jul;16(2):230.

2. Hambidge M. Human zinc deficiency. J Nutr. 2000 May;130(5S Suppl):1344S-9S.

3. Prasad AS. Effects of zinc deficiency on Th1 and Th2 cytokine shifts. J Infect Dis. 2000 Sep;182 Suppl 1:S62-8.

4. Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. Immunol Today. 1996 Mar;17(3):138-46.

5. Tanaka Y, Shiozawa S, Morimoto I, Fujita T. Role of zinc in interleukin 2 (IL-2)-mediated T-cell activation. Scand J Immunol. 1990 May;31(5):547-52.

6. Sprietsma JE. Zinc-controlled Th1/Th2 switch significantly determines development of diseases. Med Hypotheses. 1997 Jul;49(1):1-14.

7. DiPiro JT. Cytokine networks with infection: Mycobacterial infections, leishmaniasis, human immunodeficiency virus infection, and sepsis. Pharmacotherapy. 1997 Mar-Apr;17(2):205-23.

8. Yarilin AA, Belyakov IM. Cytokines in the thymus: Production and biological effects. Curr Med Chem. 2004 Feb;11(4):447-64.

9. van der Meer MJ, Sweep CG, Rijnkels CE, Pesman GJ, Tilders FJ, Kloppenborg PW, Hermus AR. Acute stimulation of the hypothalamic-pituitary-adrenal axis by

IL-1 beta, TNF alpha and IL-6: A dose response study. J Endocrinol Invest. 1996 Mar;19(3):175-82.

10. Iwakabe K, Shimada M, Ohta A, Yahata T, Ohmi Y, Habu S, Nishimura T. The restraint stress drives a shift in Th1/Th2 balance toward Th2-dominant immunity in mice. Immunol Lett. 1998 May;62(1):39-43.

11. Lepage LM, Giesbrecht JA, Taylor CG. Expression of T lymphocyte p56(lck), a zinc-finger signal transduction protein, is elevated by dietary zinc deficiency and diet restriction in mice. J Nutr. 1999 Mar;129(3):620-7.

12. Varas A, Vicente A, Romo T, Zapata AG. Role of IL-2 in rat fetal thymocyte development. Int Immunol. 1997 Oct;9(10):1589-99.

13. Kopf M, Baumann H, Freer G, Freudenberg M, Lamers M, Kishimoto T, Zinkernagel R, Bluethmann H, Kohler G. Impaired immune and acute-phase responses in interleukin-6-deficient mice. Nature. 1994 Mar 24;368(6469):339-42.

14. Moore JB, Blanchard RK, McCormack WT, Cousins RJ. cDNA array analysis identifies thymic LCK as upregulated in moderate murine zinc deficiency before T-lymphocyte population changes. J Nutr. 2001 Dec;131(12):3189-96.

15. Hunig T, Torres-Nagel N, Mehling B, Park HJ, Herrmann T. Thymic development and repertoire selection: The rat perspective. Immunol Rev. 2001 Dec;184:7-19.

16. Hosea HJ, Rector ES, Taylor CG. Zinc-deficient rats have fewer recent thymic emigrant (CD90+) T lymphocytes in spleen and blood. J Nutr. 2003 Dec;133(12):4239-42.

17. Mackall CL, Hakim FT, Gress RE. T-cell regeneration: All repertoires are not created equal. Immunol Today. 1997 May;18(5):245-51.

18. Beck FW, Prasad AS, Kaplan J, Fitzgerald JT, Brewer GJ. Changes in cytokine production and T cell subpopulations in experimentally induced zinc-deficient humans. Am J Physiol. 1997 Jun;272(6 Pt 1):E1002-7.

19. Hassan J, Reen DJ. Human recent thymic emigrants--identification, expansion, and survival characteristics. J Immunol. 2001 Aug 15;167(4):1970-6.

20. Shi HN, Scott ME, Stevenson MM, Koski KG. Energy restriction and zinc deficiency impair the functions of murine T cells and antigen-presenting cells during gastrointestinal nematode infection. J Nutr. 1998 Jan;128(1):20-7.

21. Lechner O, Dietrich H, Oliveira dos Santos A, Wiegers GJ, Schwarz S, Harbutz M, Herold M, Wick G. Altered circadian rhythms of the stress hormone and melatonin response in lupus-prone MRL/MP-fas(ipr) mice. J Autoimmun. 2000 Jun;14(4):325-33.

22. Hosea HJ, Rector ES, Taylor CG. Dietary repletion can replenish reduced T cell subset numbers and lymphoid organ weight in zinc-deficient and energy-restricted rats. Br J Nutr. 2004 May;91(5):741-7.

10. CONCLUSIONS

10.1 Discussion

The experiments described in this thesis employed various dietary treatment controls. The baseline group provided a reference point to compare the effects of the dietary treatments during growth. CTL represented "normal" growing rats, while DR controlled for the anorexia of ZD. If both ZD and DR are significantly different from CTL, but not each other, then the difference is due to the malnutrition and not specific to the zinc deficiency. If ZD is different from DR, then there is a difference specific to zinc beyond the malnutrition. In chapters 7-9, MZD was added as an additional group to represent the degree of zinc deficiency that is more prevalent in the human population. The MZD group has a reduced zinc status without anorexia, which allows for the examination of zinc deficiency without the confounding variable of malnutrition. It is difficult to interpret findings that show ZD, DR, and MZD lower than CTL, but not different from each other e.g. IL-2 production by thymocytes (chapter 9). The cytokine data (CTL>MZD=ZD=DR) could be not be explained by the serum corticosterone concentrations (CTL=MZD<ZD=DR; chapter 9). If these cytokine results were due to zinc, then a dose response (CTL>DR>MZD>ZD) would be expected, but was not seen. Perhaps the malnutrition associated with ZD is so severe that the effect of zinc deficiency can no longer be separated from the malnutrition. However, the reduced cytokine response in the MZD group could reflect reduced zinc status in the absence of malnutrition. Thus the true value of the MZD group is that it isolates the effect of zinc.

One of the first objectives of this thesis was to characterize the effects of dietary zinc deficiency on the T-cells from the thymus, spleen and blood in growing rats. We discovered a difference between the adult mouse and growing rat models of dietary zinc deficiency; there was a lack of lymphopenia relative to organ weight in the rat (chapter 5). The zinc deficient adult mouse results in wasting malnutrition, while the zinc deficient growing rat results in stunting malnutrition. This difference should be taken into account when selecting an appropriate model and subsequently extending the findings to the population of interest.

Both the young zinc deficient rat and adult mouse have the ability to recover lymphoid organ weight and cell numbers (chapter 5) (1), however, the functional ability of these cells was not determined. There appears to be a priority for recovering lymphoid tissue before body weight (chapter 5). Supplemental zinc in excess of the recommendations can result in reduced copper status (2), therefore the finding that excessive amounts of dietary zinc are not necessary for recovery of lymphoid tissue is relevant to programs aimed at treating dietary zinc deficiency.

Both the zinc deficient adult mouse and the zinc deficient growing rat have elevated $p56^{lck}$ protein levels in the thymus compared to *ad libitum* fed controls (3), but the zinc deficient growing rat did not have elevated splenic $p56^{lck}$ protein levels as observed in the zinc deficient adult mouse (4). In the present study, the $p56^{lck}$ protein levels represent the amount of protein from splenocytes and not specifically T-cells which were isolated by immunocolumns in the study with the adult mouse (4). Perhaps the $p56^{lck}$ protein levels are also elevated in the splenic T-cells of zinc

deficient growing rats, but we were not able to detect the difference due to the presence of other mononuclear cells in the spleen.

The theoretical outcome of elevated thymocyte $p56^{lck}$ is defective thymocyte maturation or increased susceptibility to apoptosis (5, 6). However, there were no differences among the dietary groups in the proportion of the thymocyte subsets, except for a higher proportion of $TCR\alpha\beta^{+}CD4^{-}CD8^{+}$ cells in ZD compared to CTL. There were no differences among the dietary treatment groups in the proportion of T-cell subsets undergoing apoptosis (Chapter 8). Future studies should examine whether the function of $p56^{lck}$ is altered by zinc deficiency. The function of $p56^{lck}$ could be evaluated experimentally by measuring the level of phosphorylated $p56^{lck}$ after stimulation of T-cells isolated from ZD, DR, MZD and CTL. Another way to assess the function of $p56^{lck}$ would be to measure a functional outcome such as proliferation or cytokine production. Isolated T-cells could be stimulated with different mitogens to determine if the TCR signaling pathway (which involves $p56^{lck}$) is altered by zinc deficiency. For example ConA stimulates via the TCR and phytohemagglutinin stimulates independent of TCR.

The zinc deficient adult mouse has been shown to have an increased proportion of $CD4^{+}CD8^{+}$ thymocytes undergoing apoptosis (7), but this is not seen in the zinc deficient growing rat (chapter 8). The increased apoptosis in the zinc deficient adult mouse thymocytes has been associated with elevated corticosterone concentrations (7). Both the zinc deficient adult mouse and the zinc deficient young rat have elevated serum corticosterone concentrations, however, by including a pair-fed group and a marginal zinc deficient group in our studies of the zinc deficient

growing rat, we have determined that the elevated steroid concentrations are not associated with increased apoptosis or decreased Th1 cytokine production (chapters 5, 8 and 9). Other components of the hypothalamus-pituitary-adrenal stress axis (serum ACTH, haptoglobin, and TNFα concentrations) were not affected by dietary zinc deficiency in the growing rat (chapter 5), thus, the cause of the elevated serum corticosterone concentrations remains to be elucidated. For studies of dietary zinc deficiency, it is necessary to house the rats in stainless steal hanging cages to reduce zinc recycling. There is some concern that the hanging cages are stressful to the animals, however, the CTL rats had serum corticosterone concentrations that are well within the normal range (chapters 5 and 9). The rats were handled everyday in order to limit the effect of handling on stress levels, and this was also reflected in the normal serum corticosterone concentrations in CTL.

Both the ZD adult mouse and growing rat models do not show major alterations in their respective thymic and splenic T-cell subsets using CD4 and CD8 cell surface labeling indicating that zinc deficiency does adversely affect a specific T-cell subset (chapter 4)(3, 8). However, the growing zinc deficient rat does have a lower proportion of splenic and blood $CD90^+$ T-cells when compared to *ad libitum* fed controls (chapter 4). In the rat, CD90 can be used to identify T-cells newly released from the thymus (9). Over time, fewer new T-cells could adversely affect the T-cell repertoire, resulting in the immunodeficiency seen with dietary zinc deficiency. The study of T-cell repertoire is also of interest in other diseases like aplastic anemia, leukemia and graft-versus-host disease in humans (10).

To explore the reason for the lower proportion of $CD90^+$ T-cells in zinc deficient rats, we used CD45RC and RT6.1 cell surface labeling along with CD90 to study the post-thymic maturation of T-cells in the spleen (chapter 7). We discovered that there were no differences among dietary treatment groups in the first two stages of post-thymic development; however, ZD, DR and MZD had a lower proportion of late thymic emigrants compared to controls (chapter 7). Future studies should investigate the kinetics of thymocyte export to determine if output is maintained or slowed down during deficiency. Along with cell surface labeling, we used DAPI to stain DNA and generate cell cycle histograms to determine the proportion of apoptotic thymic and splenic T-cell phenotypes (chapter 8). There was no evidence of increased apoptosis in the spleen and thymus among dietary treatment groups, and therefore, the decreased proportion of late thymic emigrants can not be explained by increased apoptosis in the splenic T-cells from zinc deficient rats. It has been reported that there is a higher proportion of apoptotic recent thymic emigrants in the liver of the diabetes prone BioBreeding rat compared to the diabetes resistant rats (11). Future studies should include other peripheral organs to determine if the late thymic emigrants have homed to the lymph nodes, gut associated lymphoid tissue or the liver instead of the spleen.

Zinc deficient humans have decreased peripheral blood mononuclear cell IL-2, IFNγ and TNFα production when stimulated with the T-cell mitogen phytohemagglutinin, however, there was no effect on the Th2 cytokine production (IL-4 and IL-10) (12). Splenic T-cells from zinc deficient mice that were exposed to Heligmosomoides polygyrus (a worm that would elicit a Th2 response) produced

less IL-4, IL-5 and IFNγ compared to zinc sufficient controls (8). We found that splenic T-cells from zinc deficient rats produced less IL-2 when stimulated with ConA compared to both pair-fed and *ad libitum* fed controls (chapter 9). Taken together, it appears that dietary zinc deficiency reduces the ability of both Th1 and Th2 cells to function appropriately depending on the mitogen used. The work described in this thesis is also the first to document the effects of zinc deficiency on the ability of thymocytes to produce cytokines (chapter 9). We discovered that ZD, DR and MZD can reduce the ability of thymocytes to produce IL-2 and IL-6 which are important for T-cell development (chapter 9). It is tempting to draw an association between the decreased thymocyte cytokine production and the decreased proportion of splenic late thymic emigrants observed in ZD, DR, and MZD. This potential relationship should be explored in future experiments. Although the work described in this thesis did not find an altered production of cytokines by splenocytes from DR and MZD, perhaps over a longer period of time differences would appear.

The number of animals per dietary treatment group used in the experiments described in this thesis are typical of controlled rodent experiments, which range from n=4 to n=10. These numbers are sufficient to detect differences among dietary treatment groups, for example, the lower proportion of recent thymic emigrants in ZD compared to DR and CTL (chapters 4 and 8). Flow cytometry provides a very precise measurement of cell populations because the proportions of cell subsets are based on a minimum of 10 000 observations. Despite the fact that the numerical difference in the proportion of recent thymic emigrants between ZD and DR and CTL groups is small, we are confident that an independent lab would be able to

reproduce our results because of the precision of the flow cytometry values and the reproducibility of these results in a subsequent experiment (chapters 4 and 8).

In summary, this thesis characterized the immunological effects of dietary zinc deficiency in the growing rat. Zinc deficiency results in stunting malnutrition with lymphocyte numbers and lymphoid organ weights proportional to body size. The lymphoid organ weights, cell numbers and proportion of $CD90^+$ T-cells recovered within 3 weeks of dietary repletion. The elevated serum corticosterone concentrations found in zinc deficiency were not associated with increased T-cell apoptosis or decreased Th1 cytokine production. The increased thymocyte $p56^{lck}$ protein expression was not associated with increased apoptosis; however, compared to controls, zinc-deficient rats had a higher proportion of thymic cytotoxic T-cells indicating an alteration in thymocyte development. Growing zinc deficient rats had a lower proportion of $CD90^+$ T-cells, and more specifically, fewer late thymic emigrants. Over time, the lower proportion of thymic emigrants could result in a less diverse T-cell repertoire leaving the ZD subject more susceptible to infection than CTL.

10.2 Summary of Major Contributions

- Zinc-deficient rats have lower thymus and spleen weights compared to controls; however, thymic or splenic atrophy relative to body weight is not present in zinc-deficient growing rats.
- Zinc-deficient rats have lower numbers of thymocytes and splenocytes; however, T-cell lymphopenia relative to organ weight or per µL blood is not present in zinc-deficient growing rats.
- Lymphoid organ weights, cell numbers, and proportion of $CD90^+$ T-cells can recover to control levels within 3 weeks following repletion with control diet.
- Both zinc-deficient and diet-restricted rats have higher serum corticosterone concentrations compared to controls; however, there was no difference among dietary treatment groups in ACTH, TNF-α and haptoglobin. The increased serum corticosterone concentrations are not associated with increased apoptosis or decreased Th1 cytokine production by splenocytes and thymocytes.
- Thymocyte $p56^{lck}$ protein levels are elevated in zinc deficiency. The increased $p56^{lck}$ protein levels are not associated with increased apoptosis in the thymus, but there is a higher proportion of $TCR\alpha\beta^+CD4^-CD8^+$ thymocytes in zinc-deficient rats compared to controls suggesting altered thymocyte development.
- There is a reduced proportion of $TCR\alpha\beta^+$ cells that express CD90 (recent thymic emigrants) in the blood and spleen of zinc-deficient rats.
- The lower proportion of $CD90^+$ T-cells in zinc-deficient rats is not due to increased susceptibility to apoptosis in the thymus or spleen.

- The lower proportion of $CD90^+$ T-cells in zinc-deficient rats is due to a lower proportion of late thymic emigrants ($TCR\alpha\beta^+CD90^+CD45RC^+RT6.1^+$), but the first two stages of post-thymic development are not affected. The lower proportion of late thymic emigrants can not be explained by increased removal via apoptosis.
- There is a higher proportion of cytotoxic thymocytes, but a lower proportion of New Cytotoxic T-cells in the spleen compared to control and marginally zinc-deficient groups, suggesting that export of New Cytotoxic T-cells from the thymus is more limited than New helper T-cells.

10.3 Contribution to Present State of Knowledge

- An examination of the interrelationships among the hypothalamus-pituitary-adrenal stress axis, $p56^{lck}$ protein levels, dietary zinc deficiency and repletion, and their effects on T-lymphocyte maturation and apoptosis (programmed cell death).
- Characterization of the effects of dietary zinc deficiency on T-cell development in the growing rat, which will hopefully stimulate further research using this model. The work described herein has dispelled the hypothesis that elevated corticosterone concentrations play a major role in the immunodeficiency associated with dietary zinc deficiency, but it does provide support for the hypothesis that increased $p56^{lck}$ expression alters thymocyte development reducing the functional ability of T-cells in dietary zinc deficiency.
- Apoptosis was found not to contribute to the lymphopenia of dietary zinc deficiency. In fact, the experimental results reported in this thesis dispute the

concept that dietary zinc deficiency in the growing rat results in lymphopenia, suggesting that the immunodeficiency of zinc deficiency is due mainly to a decrease in T-cell function and not due solely to reduced cell numbers.

There are a lower proportion of recent thymic emigrants in dietary zinc deficiency. Overtime, fewer recent thymic emigrants could reduce the diversity of the T-cell repertoire leaving the zinc-deficient subject more susceptible to infection.

10.4 Implications

- Recovery from immune dysfunction due to dietary zinc deficiency during growth is possible; therefore, programs aimed at increasing the availability of zinc to children, particularly in developing countries, should be developed and funded. Supplemental zinc concentrations above recommended dietary levels are not necessary for recovery of lymphoid tissue, which limits the danger of copper deficiency.
- Even a mild zinc deficiency results in altered immune function; however, clinical methods to reliably diagnose zinc deficiency are not available. The development of methods to diagnose marginal zinc deficiency is essential so that children can be treated and live healthy lives.
- The immune measures in the blood of growing rats reflect the immune measures in the spleen of growing rats, which provides some evidence that the blood can be used with confidence in human trials of immune function.

10.5 Limitations

- There is an imbalance in the proportion of recent thymic emigrants in dietary zinc deficiency; however, we did not specifically measure the function of these cells to determine if their function is significantly different from other phenotypes which would explain an overall change in the ability of the immune system to fight off infection. We also did not measure the diversity of the T-cell repertoire to determine whether it is lowered by dietary zinc deficiency.
- This work did not measure the activity of $p56^{lck}$ in T-cells from zinc deficient rats directly, so it remains unclear whether the increased expression of this signaling protein results in increased activity.
- Severe dietary zinc deficiency (<1 mg zinc/kg diet) is extremely rare in the human population, however, we were able to show that even a mild zinc deficiency that does not affect growth can alter immune function.
- The rat is unique in that CD90 can be used as a marker of recent thymic emigrants, but this also limits the relevance of this work to humans.
- Despite the fact that the experiments described in this thesis did not see T-cell lymphopenia relative to organ weight or per µl blood, it remains unclear whether a lower absolute number of lymphocytes in zinc deficiency compared to controls would result in increased vulnerability to infections. The ideal absolute number of T-cells to have a full repertoire to fight off possible infections is not known and was not addressed by the experiments described in this thesis.

10.6 Future Research

- Future studies should continue to address the interactions among dietary zinc deficiency, age, maturity of the immune system and species differences.
- The question remains whether the elevated $p56^{lck}$ levels in dietary zinc-deficiency results in altered function of the T-cells. It is also not known whether the elevated $p56^{lck}$ protein levels are due to increased levels in one particular T-cell subset or across all T-cell subsets. Future studies should use flow cytometry to sort or isolate individual T-cell phenotypes to address these questions further.
- An examination at the cellular level into whether there is an association between CD90 cell surface expression and $p56^{lck}$ protein levels/function in dietary zinc deficiency should be investigated.
- The repertoire of T-cells from zinc deficient rats should be examined to determine whether it is limited by the deficiency.
- Future studies of dietary zinc deficiency should also include T-cells isolated from lymph nodes, peyer's patches, and liver in their investigations to determine whether there are fewer recent thymic emigrants present in these peripheral lymphoid organs as we have seen in the spleen and blood.
- An examination of the ability of the immune system in the zinc deficient growing rat under an *in vivo* pathogenic challenge should also be examined. The immune system has built in redundancy, and it would be of interest to determine if the zinc deficient growing rat is more susceptible to infection or if

other parts of the immune system would be able to compensate for the decreased T-cell function.

10.7 Literature Cited

1. Fraker PJ, DePasquale-Jardieu P, Zwickl CM, Luecke RW. Regeneration of T-cell helper function in zinc-deficient adult mice. Proc Natl Acad Sci U S A. 1978 Nov;75(11):5660-4.

2. Jamieson JA, Taylor CG, Weiler HA. Marginal zinc deficiency exacerbates bone lead accumulation and high dietary zinc attenuates lead accumulation at the expense of bone density in growing rats. Toxicol Sci. 2006 Jul;92(1):286-94.

3. Moore JB, Blanchard RK, McCormack WT, Cousins RJ. cDNA array analysis identifies thymic LCK as upregulated in moderate murine zinc deficiency before T-lymphocyte population changes. J Nutr. 2001 Dec;131(12):3189-96.

4. Lepage LM, Giesbrecht JA, Taylor CG. Expression of T lymphocyte p56(lck), a zinc-finger signal transduction protein, is elevated by dietary zinc deficiency and diet restriction in mice. J Nutr. 1999 Mar;129(3):620-7.

5. Levin SD, Anderson SJ, Forbush KA, Perlmutter RM. A dominant-negative transgene defines a role for p56lck in thymopoiesis. EMBO J. 1993 Apr;12(4):1671-80.

6. Di Somma MM, Nuti S, Telford JL, Baldari CT. p56lck plays a key role in transducing apoptotic signals in T cells. FEBS Lett. 1995 Apr 17;363(1-2):101-4.

7. King LE, Osati-Ashtiani F, Fraker PJ. Apoptosis plays a distinct role in the loss of precursor lymphocytes during zinc deficiency in mice. J Nutr. 2002 May;132(5):974-9.

8. Shi HN, Scott ME, Stevenson MM, Koski KG. Energy restriction and zinc deficiency impair the functions of murine T cells and antigen-presenting cells during gastrointestinal nematode infection. J Nutr. 1998 Jan;128(1):20-7.

9. Hosseinzadeh H, Goldschneider I. Recent thymic emigrants in the rat express a unique antigenic phenotype and undergo post-thymic maturation in peripheral lymphoid tissues. J Immunol. 1993 Mar 1;150(5):1670-9.

10. Plasilova M, Risitano A, Maciejewski JP. Application of the molecular analysis of the T-cell receptor repertoire in the study of immune-mediated hematologic diseases. Hematology. 2003 Jun;8(3):173-81.

11. Iwakoshi NN, Goldschneider I, Tausche F, Mordes JP, Rossini AA, Greiner DL. High frequency apoptosis of recent thymic emigrants in the liver of lymphopenic diabetes-prone BioBreeding rats. J Immunol. 1998 Jun 15;160(12):5838-50.

12. Beck FW, Prasad AS, Kaplan J, Fitzgerald JT, Brewer GJ. Changes in cytokine production and T cell subpopulations in experimentally induced zinc-deficient humans. Am J Physiol. 1997 Jun;272(6 Pt 1):E1002-7.

11. APPENDIX

11.1 TCRαβ and CD90 Fluorescent Labeling in Rat Thymus.

For the experiments described in this thesis, it was of interest to characterize T-cells based on their CD90 and TCRαβ cell surface expression using flow cytometry. In the thymus, approximately 95% of thymocytes express CD90. As T-cells develop, TCRαβ expression on thymocytes change from null, to low, to high. During immunophenotyping, it is typical to add all directly labeled antibodies to samples at the same time. However, when we added CD90 and TCRαβ simultaneously, we noticed that we did not receive the typical three peaks of TCRαβ expression. We hypothesized that CD90 competes with TCRαβ in the thymus; therefore, we conducted an experiment to test this theory.

Thymocytes were treated using the following protocol:

1. Sterilize all instruments overnight in 70% ethanol.
2. Terminate rat (~4 weeks old) by CO_2 asphyxiation and decapitation.
3. Remove thymus aseptically (pour 70% ethanol on animal prior to opening cavity and use sterile scissors and tweezers), and place in petri dish containing Hank's buffered saline containing10 mmol/L HEPES and 4% fetal bovine serum, pH 7.4 (buffer 1).
4. In cell culture hood, push cells through nylon mesh using barrel of sterile syringe into a Petri dish containing 10 mL buffer 1. Transfer cell suspension to 50 mL conical tube through a sterile filter (70 microns). Rinse plate twice

with 10 mL buffer 1. Spin sample at 300 g (1200 rpm) for 8 minutes to pellet cells (4°C). Discard supernatant.

***It is important to keep the staining vessels partially submerged in an ice bath and to include sodium azide in the staining buffer. Modulation and internalization of surface antigens can occur as a result of antibody crosslinking, which can produce a loss of fluorescence intensity. Low temperature and the presence of sodium azide prevent this phenomenon by inhibiting metabolic activity.

5. Resuspend pellet in 10 mL buffer 1.
6. Count using hemacytometer and trypan blue.
    - Transfer 0.5 mL of 0.4% Trypan blue solution and 0.3 mL of Hank's buffered saline to a microcentrifuge tube. Add 10 µL cell suspension (equals a dilution factor of 81).
    - Mix thoroughly and allow to stand for 5 minutes. Load onto hemocytometer and count cells in the four corner squares (separate counts for blue and clear cells). Should have between 20-50 cells/square, otherwise use a different dilution factor.
        - Cells per mL = average count per square x dilution factor x 10^4
        - Cell viability (%) = total unstained cells/total cells x 100.
7. Adjust concentration of cell suspension to 1 x 10^6 cells/mL.
    - ie. (2 x 10^7 cells/mL)(X) = (1 x 10^6 cells/ml)(10mL)
    - X=0.5 mL cell suspension + 9.5 mL buffer 1.
8. Transfer 1 mL of cell suspension (1x 10^6 cells/mL) into 12x15-mm round-bottom test tubes (staining tubes).

9. Centrifuge cell suspension 8 minutes at 300 g, 4˚C, and discard supernatant. Resuspend cell pellet in 100 µL phosphate buffered saline containing 0.1% sodium azide and 1% fetal bovine serum (label buffer).

***Thymocytes were incubated with anti-rat monoclonal antibodies directly conjugated to either PE or PerCP in the following combinations: TCRαβ alone, CD90 alone, TCRαβ with CD90 at the same time, TCRαβ first followed by CD90, and CD90 first followed by TCRαβ.

10. Add 10 µL of diluted (2.5 µL of antibody + 7.5 µL label buffer) TCRαβ-PE, or CD90-PerCP to appropriate tubes.
11. Incubate for 30 minutes at 4°C.
12. After incubation, add 2 mL label buffer, spin at 300 g, 4˚C for 8 minutes and discard supernatant. Resuspend pellet in 100 µL label buffer.
13. Add 10 µL of diluted TCRαβ-PE, or CD90-PerCP to appropriate tubes.
14. Incubate for 30 minutes at 4°C.
15. Add 2 mL label buffer, spin at 300 g for 8 minutes and discard supernatant.
16. Resuspend pellet in 1 mL 1% paraformaldehyde (made fresh each week). Store at 4˚C until analysis on flow cytometer.

Antibodies: Anti-rat monoclonal antibodies against TCRαβ (PE label, clone R73, isotype mouse $IgG_{1,k}$) and CD90 (PerCP label, Thy1.1, clone OX-7, isotype, mouse $IgG_{1,k}$) were obtained from BD Pharmingen (Mississauga, ON).

Flow cytometric analysis was performed on a Beckman Coulter EPICS ALTRA (Beckman Coulter, Mississauga, ON) high-speed cell sorter with laser excitation tuned to 488 nm (65 mW). Forward versus side scatter histograms were

used to identify lymphocytes. The fluorescence signals were separated with the standard dichotic long pass filters provided with the instrument and detected through 575 nm (PE) and 675 nm (PerCP) bandpass filters. Flurochrome-isotype matched controls were prepared to assess autofluorescence and nonspecific binding, while single color samples were employed to adjust color compensation.

The results are shown in Figure 11.1.1. When CD90 and TCR$\alpha\beta$ antibodies are added simultaneously (c-d), or when CD90 is added before TCR$\alpha\beta$ (g-h), we do not see the typical three peaks associated with null, low, and high TCR$\alpha\beta$ expression in thymocytes (a).

We conclude that CD90 antibodies competes with TCR$\alpha\beta$ antibody binding, and therefore, TCR$\alpha\beta$ antibodies should be added before CD90 antibodies in order to visualize the low TCR$\alpha\beta$ expression in thymocytes (e-f). In splenocytes CD90 competition is not a problem because TCR$\alpha\beta$ is highly expressed on mature T-cells.

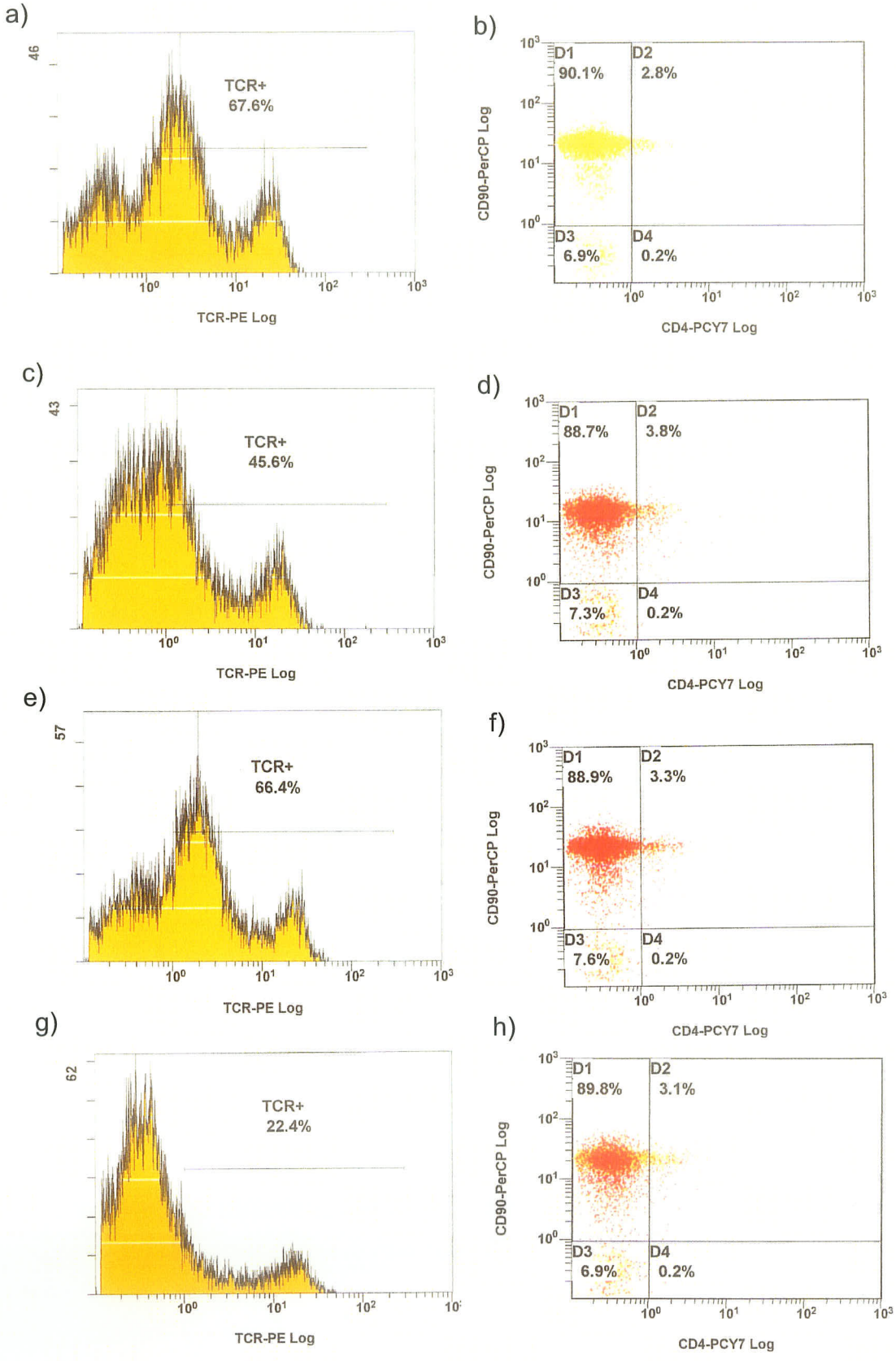

a)
46
TCR+
67.6%
TCR-PE Log
b)
D1
90.1%
D2
2.8%
D3
6.9%
D4
0.2%
CD90-PerCP Log
CD4-PCY7 Log
c)
43
TCR+
45.6%
TCR-PE Log
d)
D1
88.7%
D2
3.8%
D3
7.3%
D4
0.2%
CD90-PerCP Log
CD4-PCY7 Log
e)
57
TCR+
66.4%
TCR-PE Log
f)
D1
88.9%
D2
3.3%
D3
7.6%
D4
0.2%
CD90-PerCP Log
CD4-PCY7 Log
g)
62
TCR+
22.4%
TCR-PE Log
h)
D1
89.8%
D2
3.1%
D3
6.9%
D4
0.2%
CD90-PerCP Log
CD4-PCY7 Log

Figure 11.1.1 TCRαβ and CD90 staining in rat thymocytes. TCRαβ alone (a); CD90 alone (b); TCRαβ and CD90 added simultaneously (c-d); TCRαβ added first followed by CD90 (e-f); CD90 added first followed by TCRαβ (g-h).

11.2 Fixing and Permeabilization of Thymocytes and Splenocytes for Simultaneous Staining of Cell Surface Markers and DNA.

One of the objectives of this thesis was to determine the proportion of apoptosis in T-cell sub-populations among zinc-deficient, diet-restricted, marginally zinc-deficient and control growing rats. In order to achieve this objective, it was necessary to label thymocytes and splenocytes with cell surface markers to identify their phenotype while simultaneously staining DNA to identify cells in the hypodiploid region of the cell cycle.

The general procedure for cell surface and intra-cellular staining is as follows: cell surface antigens are labeled first, and then the cells are fixed (to maintain cell surface staining) followed by a permeabilization step to allow a fluorescent dye (ie. DAPI) to enter the cell and bind to the DNA.

Ethanol both fixes and permeabilizes cells; however, it obliterates the PerCP fluorochrome and the dull TCR$\alpha\beta$ peak in thymocyes (Figure 11.2.1a-b). Cells can also be fixed for a short time in 1% paraformaldehyde followed by permeablization with 0.1% tween, which does not affect the PerCP fluorochrome and the dull TCR$\alpha\beta$ peak in thymocytes (Figure 11.2.2 a-b). Special care must be taken when fixing with paraformaldehyde in that the fixation is not so extensive that the fixative begins crosslinking the DNA, which would affect the ability of the fluorescent dye to bind to DNA. We compared these two methods of fixing/permeabilizing using the following protocol:

1. Steps 1-7 as described in 11.1.
2. Transfer 1 x 10^6 cells into 4 mL culture tubes.

3. Add cell culture medium (RPMI-1640 containing 10 mmol/L HEPES, 10 mmol/L sodium bicarbonate, 1 mmol/L sodium pyruvate, 2 mmol/L glutamine, 0.1 mmol/L non-essential amino acids, 50 µmol/L mercaptoethanol, 5% fetal bovine serum, pH 7.4) to 840 µL.
4. Cells were incubated with or without 1 µmol/L dexamethasone (DEX)=0.4 µg/mL for 7 hours.
    - Add 990 µL cell culture media to 10 µL DEX at a concentration of 25 mg/mL (final concentration=0.25 mg DEX/mL).
    - Take 10 µL of 0.25 mg DEX/mL and add 990 µL cell culture media (final concentration=0.0025 mg DEX/mL).
    - For 0.4 µg DEX/mL add 160 µL of 0.0025 mg DEX/mL to 1 x 10^6 cells in 840 µL cell culture media.
5. After incubation, add 2 mL phosphate buffered saline, centrifuge 5 minutes at 350 g, 4˚C, and discard supernatant by rapidly decanting.
6. Resuspend in 100 µL phosphate buffered saline containing 0.1% sodium azide and 1% fetal bovine serum (label buffer). Add 10 µL of appropriately diluted TCR-PE or CD90-PerCP antibodies (as described in 11.1).
7. Incubate for 30 minutes at 4˚C.
8. After incubation, add 2 mL label buffer, centrifuge 5 minutes at 350 g, 4˚C, and discard supernatant by rapid decanting.
9. a) Ethanol treatment: Resuspend pellet in 300 µL phosphate buffered saline-50% fetal bovine serum and add 700 µL 70% ethanol dropwise while gently mixing (for a final concentration of 50% ethanol). Incubate overnight 4˚C.

b) Paraformaldehyde treatment: Add 1 mL 1% paraformaldehyde and mix. Incubate 30 minutes at 4˚C, and add 2 mL phosphate buffered saline. Centrifuge cells 5 minutes at 350 g, 4˚C. Remove supernatant by rapid decanting. Add 1 mL permeabilization solution (0.1% Tween) to cell pellet. Mix gently and incubate 15 minutes at room temperature. Add 2 mL phosphate buffered saline, centrifuge at 350 g, 4˚C for 5 minutes and rapidly decant. Resuspend cells in 1 mL phosphate buffered saline, and store overnight at 4˚C.

10. The following morning, wash ethanol treated cells by adding 2 mL phosphate buffered saline, centrifuge at 350 g, 4˚C for 5 minutes and rapidly decant.
11. Add 990 µL phosphate buffered saline to 10 µL of 10 mg DAPI/mL (final concentration= 100 µg DAPI/mL). Take 10 µL of 100 µg DAPI/mL and add to 990 µL phosphate buffered saline (1 µg/mL).
12. Add 110 µL of 1 µg/mL DAPI solution (final concentration 0.1 µg/mL) to appropriate tubes.
13. Analyze on flow cytometer as described in 11.2

We found that we were able to maintain cell surface marker staining using the fixation with 1% paraformaldehyde followed by permeabilization with 0.1% tween (Figure 11.2.2a-b), but not using ethanol. We were able to stain DNA with both permeablization methods (Figure 11.2.1c-d and 11.2.2c-d). However, only cells that were fixed in ethanol showed DNA in the hypodiploid region of the cell cycle histogram after induction of apoptosis using dexamethasone (Figure 11.2.1d and

11.2.2d). We conclude that although the 0.1% tween procedure permeabilized cell membranes enough to allow entry of DAPI into the cell, the cell membrane was not permeabilized enough to allow the fragmented DNA to escape to create cells with low levels of DNA, which can then be identified as apoptotic on the cell cycle histogram. Thus, we concluded that ethanol treatment is essential to identify apoptotic cells using cell cycle histograms.

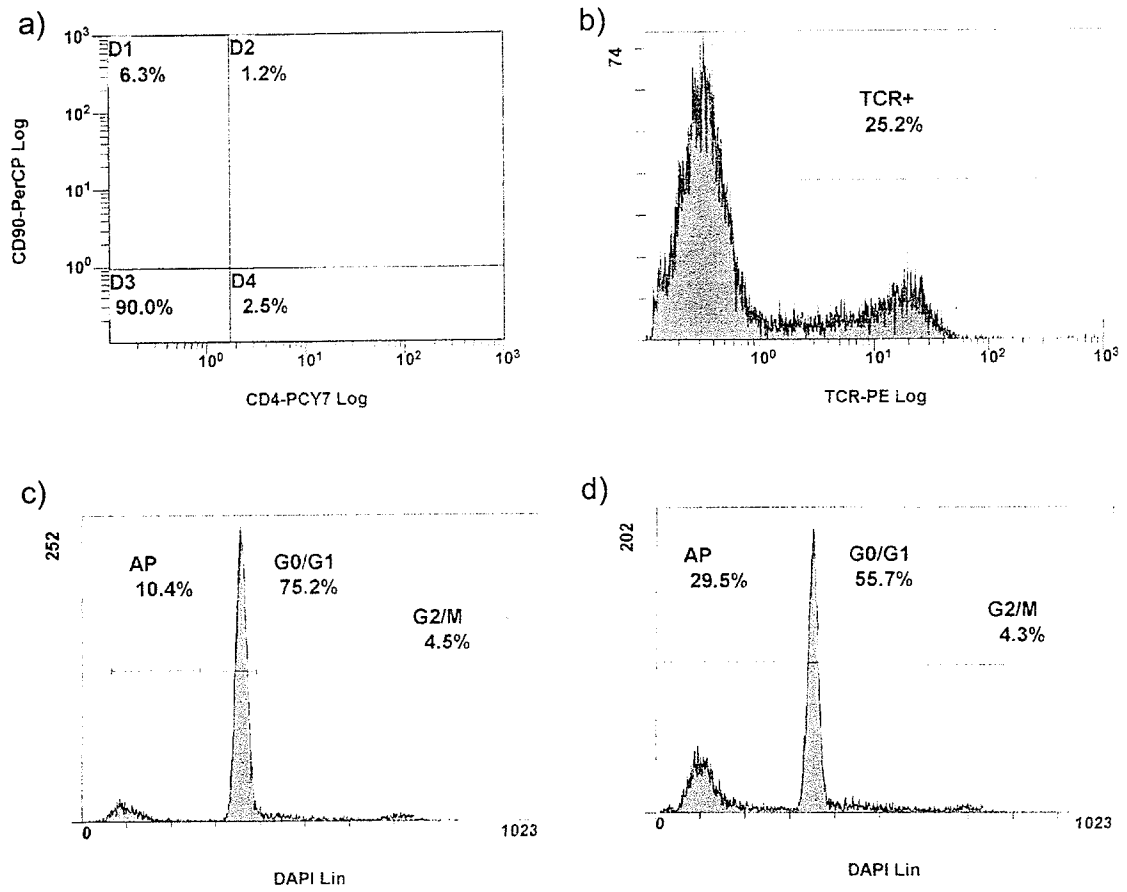


Figure 11.2.2 Fixing and permeabilizing thymocytes with ethanol. CD90-PerCP staining (a); TCRαβ-PE staining (b); DAPI staining after 6 h, no dexamethasone (c); DAPI staining after 6 h with 1 µmol/L dexamethasone (d).

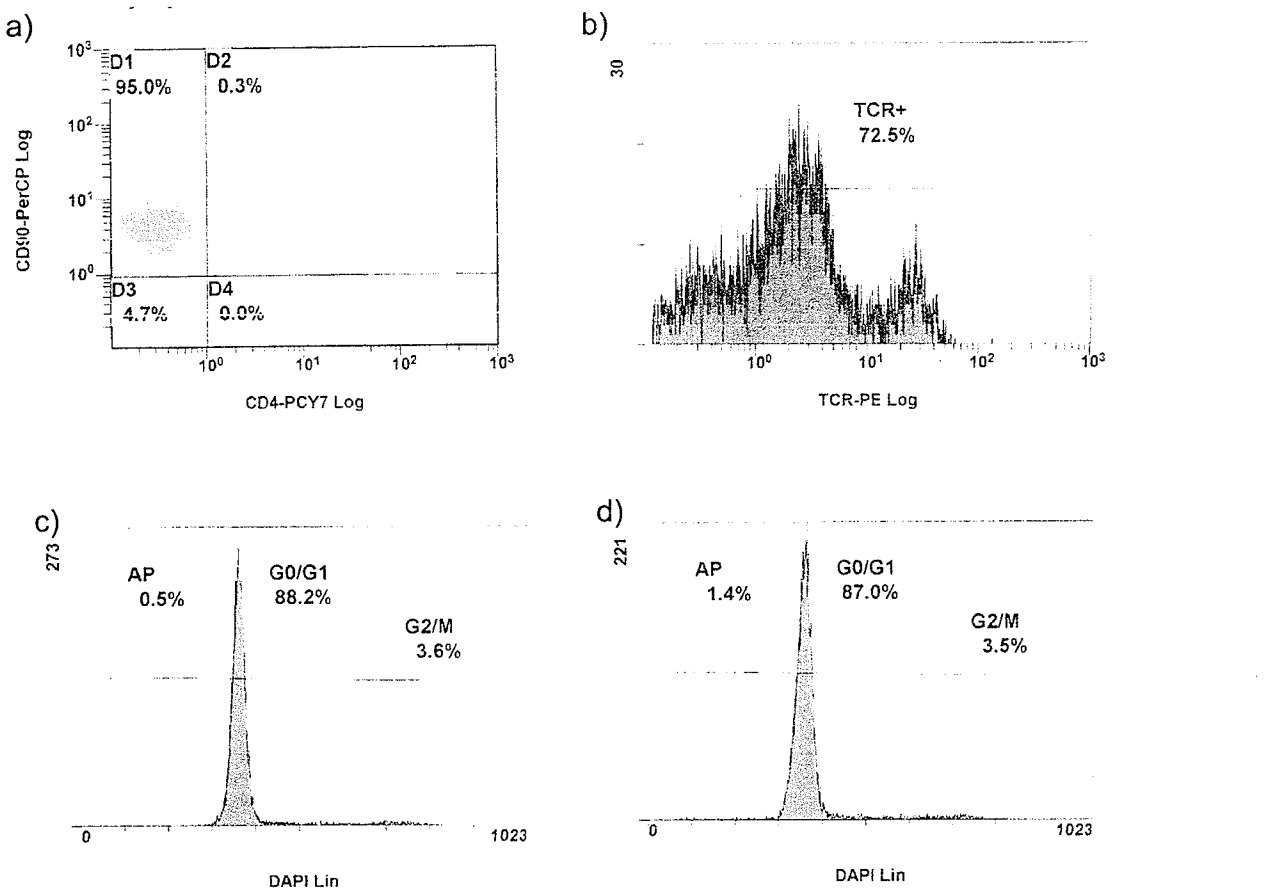


Figure 11.2.3 Fixing thymocytes with 1% paraformaldhyde for 30 minutes followed by permeabilizing with 0.1% tween. CD90-PerCP staining (a); TCRαβ-PE staining (b); DAPI staining after 6 h, no dexamethasone (c); DAPI staining after 6 h with 1 µmol/L dexamethasone (d).