MURINE ZINC DEFICIENCY ALTERS T-LYMPHOCYTE SUBPOPULATIONS AND GENE EXPRESSION OF CHEMOKINES AND CYTOKINES IN THE GUT-ASSOCIATED LYMPHOID TISSUES

By

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Zinc deficiency alters lymphopoeisis and T_H1/T_H2 cytokine balance. Although these cytokine changes may alter lymphocyte subpopulation distribution or homing, no studies have examined this outcome. BALB/c mice (4-week old) were fed a zinc-deficient (ZD, <1 mg Zn/kg), zinc-adequate (ZA, 27 mg Zn/kg), or pair-fed (PF) diet for 9 weeks. Percent baseline weight did not differ among the 3 groups in this T_H2-dominant mouse strain; therefore, PF was dropped from further analyses. Serum zinc (ug/mL) decreased with progressive zinc deficiency and at week 9 was 0.7 ± 0.1 (ZA) versus. 0.3 ± 0.2 (ZD, mean ± SEM, *P* = 0.001). Gut-associated lymphoid tissue (GALT) phenotypes were evaluated at 3, 6, and 9 weeks by flow cytometry with anti-CD3, CD8ß, TCRγδ, CD4, and CD28 antibodies. ZD $CD3^+$ colonic intraepithelial lymphocytes (cIEL) normalized to the ZA group increased with progressive zinc deficiency (*P* = 0.06), and the percentage of $CD3^+$ T cells was higher at week 9 in ZD (43 ± 4%) versus

ZA (29 ± 3%, $P = 0.04$) mice. $CD3^+$ $CD8ß^+$ $TCR\gamma\delta^-$ cIELs were elevated at all time points in ZD mice ($P = 0.008$) and may account for the T-cell increase. This cell population was lower at all time points in lymphocytes isolated from Peyer's patches ($P < 0.01$). Peyer's patch $CD3^+$ $TCR\gamma\delta^+$ $CD8\beta^-$ were elevated as a percentage of total cells ($P < 0.05$) and as a percentage of $CD3^+$ T cells ($P = 0.002$). $CD4^+$ populations and subpopulations were lower as a percentage of $CD3^+$ T cells in ZD versus ZA in cIEL ($CD4^+$, $P = 0.02$) and Peyer's patches ($CD4^+$ $CD8^+$, $P = 0.003$). Populations expressing $CD28^+$ decreased as a percentage of $CD3^+$ T cells in Peyer's Patches ($CD3^+$ $CD28^+$ $CD4^-$ $CD8\beta^+$, $P < 0.05$), cIEL ($CD3^+$ $CD4^+$ $CD28^+$, $P < 0.001$), and messenteric lymph nodes ($CD3^+$ $CD28^+$, $P = 0.04$) in ZD mice. At week 9, colonic inflammatory cytokine and receptor transcript abundance was measured using a microarray followed by q RT-PCR for confirmation. Microarray analysis indicated that CCL25 (TECK) was overexpressed in ZA whereas IL-18 was overexpressed in ZD colon. q RT-PCR confirmed that normalized CCL25 mRNA levels were different between diet groups, whereas IL-18 mRNA levels were unchanged. However, IL-18 transcript levels positively correlated with the percentage of ZA and ZD $CD3^+$ $CD8ß^+$ $TCR\gamma\delta^-$ cells. These data suggest that ZD alters GALT lymphocyte subpopulation distribution, $CD28^+$ populations, and a chemokine ligand important in lymphocyte homing.

INTRODUCTION

Zinc, a mineral that is important in the maintenance of health, functions in the body to support cell growth, cell replication, gene expression, protein metabolism, lipid metabolism, and immune function, and is also an essential cofactor for more than 200 enzymes. The body contains between 1.5 and 2.5 g of zinc, and requires approximately 8 to 11 mg/day (1). Early studies in the Middle East identified the relationship between zinc deficiency, short stature and subsequent susceptibility to infectious diseases (2,3). The initial belief that zinc deficiency was isolated to malnourished individuals in underdeveloped countries was abolished when later studies in the United States revealed that pregnant women, low-income children, and elders were also at risk for zinc deficiency (4-9). In addition, individuals suffering from chronic illnesses also were found to have zinc deficiency further exacerbating their conditions (4,10). These zinc-deficient individuals are therefore at risk for developing complications resulting in an impairment of their immune responses and a general decline in health status.

Many studies link zinc deficiency with increased diarrhea in mice and humans, suggesting disturbance of the intestinal barrier; however, little is known of the impact of zinc deficiency on mucosal immunity (4,11,12). Zinc deficiency is associated with a systemic imbalance between T_H1 (IL-2, IFN-γ, TNF-α, and IL-12) and T_H2 (IL-3, IL-4, IL-5, IL-6, IL-10, and IL-13) cytokines (13,14). Changes in cytokines, chemokines, and/or their receptors, if perpetuated into the intestinal

tissue, may be one mechanism for promoting disturbances of immune populations within the intestinal mucosa. Chemokines, cytokines, and their receptors are essential in gut-associated lymphoid tissue (GALT) maintenance and recruitment of cells during an immune response. Zinc deficiency is also associated with alterations of peripheral, thymocyte, and splenocyte cell populations (4,15-17). Mice exposed to zinc deficiency have been shown to have fewer pre-T, pre-B, and alterations in T-helper to T-cytotoxic cell ratios (15,16). Alterations in cytokine responses and lymphocyte populations of ZD mice may inhibit the ability of these mice to maintain and protect their GALT and lead to the development of inflammation.

The objective of this study was to examine the effects of a progressive zinc deficiency on GALT lymphocyte populations and gene expression of cytokines and chemokines in mouse colonic tissue. We propose that zinc status affects the lymphocyte populations and balance of chemokines, cytokines and/or their receptors, important in maintenance of the GALT. The mucosal immune system is a natural barrier and a major route of entry for invading pathogens; therefore, maintaining its integrity is critical for protection of the body from intestinal flora and potential pathogens. A clear understanding of the changes that occur in the GALT during zinc deficiency, including cytokines, chemokines, and their receptors, and cellular populations may suggest mechanisms through which immune function is altered to allow susceptibility to infectious diseases.

LITERATURE REVIEW

What is GALT?

The gut-associated lymphoid tissue (GALT) is composed of the lymphoid rich regions including the mesenteric lymph nodes, Peyer's patches, epithelium, and the lamina propria. GALT contains 10^6 lymphocytes/g tissue as reviewed by Jabbar et al. (18) making it the largest lymphoid organ in the body, with total lymphocytes outnumbering even that found in the spleen. In healthy individuals, the GALT is responsible for ensuring that an adequate immune response is generated following a pathogenic exposure and at the same time preventing the many non-pathogenic dietary antigens, which are ingested daily, from evoking an immune response. The epithelium (composed of individual epithelia) covers finger-like projections that protrude into the lumen and enhance the surface area of the intestine and allow for greater absorbative capacity. The epithelium, a single layer of cells, forms a barrier of protection from the external environment and protects the adult human mucosal surface, which extends 200 to 300m^2 (19). Along the villi, interspersed between the epithelial cells at a 6 to 10:1 (epithelial:intraepithelial cell ratio) are lymphocytes called intraepithelial lymphocytes, usually $CD8^+$ T cells (18). Epithelial cells have absorbative capacity, antigen-presenting capabilities, and transport IgA (the major protective gut antibody) into the lumen. At the base of the villi are crypts, containing stem cells that will develop into either epithelial, goblet (mucus-secreting), or paneth

cells (which secrete antimicrobial agents). The lamina propria is beneath the epithelial cells, and is a dense network of lymphatic and blood vessels. This area contains dendritic cells, macrophages, plasma cells and their precursor B cells, and $CD4^+$ and $CD8^+$ lymphocytes. Below the lamina propria are several muscle layers that provide structure and mechanical function, and are important for peristalsis. Peyer's patches are present throughout the intestine, although they are present in higher numbers in the small intestine. Peyer's patches are lymph node-like structures and are the sites for the initiation of immune responses within the small intestine. Peyer's patches contain zones of T cells and B cells, and interactions between these two types of cells occur in areas called germinal centers. Cellular populations found within the Peyer's patches include $CD4^+$ and $CD8^+$ T cells, B cells, macrophages, and dendritic cells. The epithelial side of the Peyer's patch contains specialized epithelial cells called microfold cells (M cells), which do not secrete mucus and allow for more direct contact with particles in the lumen of the intestine. The intestines drain into a series of lymph nodes called the mesenteric lymph nodes. Like the Peyer's patches, mesenteric lymph nodes have T- and B-cell zones and germinal centers. Mesenteric lymph nodes function to filter antigen out of the lymph and are composed of T and B cells, macrophages, immature dendritic cells and mature dendritic cells. Each of these GALT tissues has a specific function to protect the mucosal barrier from invasion of pathogens.

Both the innate and adaptive branches of the immune system are represented in the GALT and play important roles in the protection of the

mucosal barrier. The innate, first line, of defense includes the normal peristalsis or movement of food through the digestive tract, digestive secretions (enzymes, gastric secretions, bile, and mucus), the epithelial barrier itself, phagocytes (macrophages and neutrophils), natural killer cells, and complement proteins. These defense mechanisms are already present or available within hours after antigenic exposure. The adaptive components of the immune system take longer (days) to provide protection but provide a stronger, more specific response following an antigenic event. Components of the adaptive immune system include the B lymphocytes, which develop into antibody secreting plasma cells, and T lymphocytes, which differentiate into T helper ($CD4^+$), T cytotoxic ($CD8^+$), or suppressor/regulatory T cells. A fraction of these T, B, and plasma cells will become memory cells, which enable the adaptive immune responses to provide a faster and amplified response upon repeated exposure to the same antigen. Whereas the innate immune response is effective at nonspecific clearance of a large number of challenging agents, the adaptive immune components are responsible for antigen specific elimination of pathogens.

The innate and adaptive immune systems work together to remove pathogens from the GALT. Pathogens that are able to penetrate the epithelial surface will encounter cells of the innate immune system such as macrophages and neutrophils, which will release chemokines, cytokines, and complement factors in an attempt to control an infection. The chemokines, cytokines, and complement factors will recruit other innate system cells into the area or opsonize the pathogen to make it more easily phagocytosed. If this local

inflammatory response is not able to clear the pathogen, the specific immune response will take over. The innate response will not be covered in depth here because the cell populations evaluated in this study are a part of the adaptive immune responses.

The adaptive immune response begins when an antigen, unable to be cleared by the innate response, is taken up by an antigen-presenting cell-usually a resident immature dendritic cell. Weiner et al. (20) demonstrated that particulate antigens are preferentially taken up by the M cells (overlying Peyer's patches); whereas, soluble antigens are taken up either by the villus epithelial cells or by passing through the tight junctions between epithelial cells (paracellular movement). Furthermore, Niess et al. (21) showed that dendritic cells have the capacity to sample luminal contents by extending tentacle-like projections across the basolateral membrane between the tight junctions via a chemokine receptor-CX3CR1, dependent process. M cells can endocytose antigens and transport them across the cell to antigen-presenting cells and lymphocytes within the Peyer's patches. The antigen presenting cell (dendritic cells or macrophages) will then take up antigen either by pinocytosis or phagocytosis. Naïve B and T cells enter the Peyer's patches (which do not have afferent lymphatics) and lamina propria via interaction of L-selectin and α4β7 on their surface with mucosal adressin cellular adhesion molecule 1 (MAdCAM-1) on the blood vessel endothelium (22). Within the Peyer's patches antigen is presented by the antigen-presenting cell to B and T lymphocytes which are then activated, proliferate, and travel to the lamina propria and eventually to the

mesenteric lymph nodes. In addition to entering the lamina propria by way of the Peyer's patches, antigen can also enter the lamina propria via the villus epithelium directly or paracellularly to interact with antigen presenting cells (macrophages or dendritic cells). The epithelial cells themselves can present this antigen to antigen presenting cells residing in the lamina propria (23). Antigen presenting cells within the lamina propria activate lymphocytes present. Activated lymphocytes and antigen-presenting cells travel from the lamina propria and enter the mesenteric lymph nodes through the afferent lymphatic vessels. The organization of the mesenteric lymph nodes promotes interactions between antigen presenting cells and T cells, B cells and antigen, and activated T cells and antigen exposed B cells. The lymphocytes differentiate and proliferate within the mesenteric lymph node, leave through the efferent lymphatics, and eventually dump back into the bloodstream at the thoracic duct (Figure 1).

Naïve T and B lymphocytes travel to lymphoid organs (such as mesenteric lymph nodes and Peyer's patches); whereas, antigen-activated T cells can migrate to non-lymphoid sites. Several studies have shown that GALT derived dendritic cells generate $CD4^+$ and $CD8^+$ cells that preferentially home back to the gut via expression on their surface of chemokine receptors that are specific for ligands expressed within the GALT (24-27). $CD4^+$ T cells activated within mesenteric lymph nodes expressing chemokine receptor 9 (CCR9, whose ligand chemokine ligand 25 is expressed by epithelial cells) and α4β7 along with

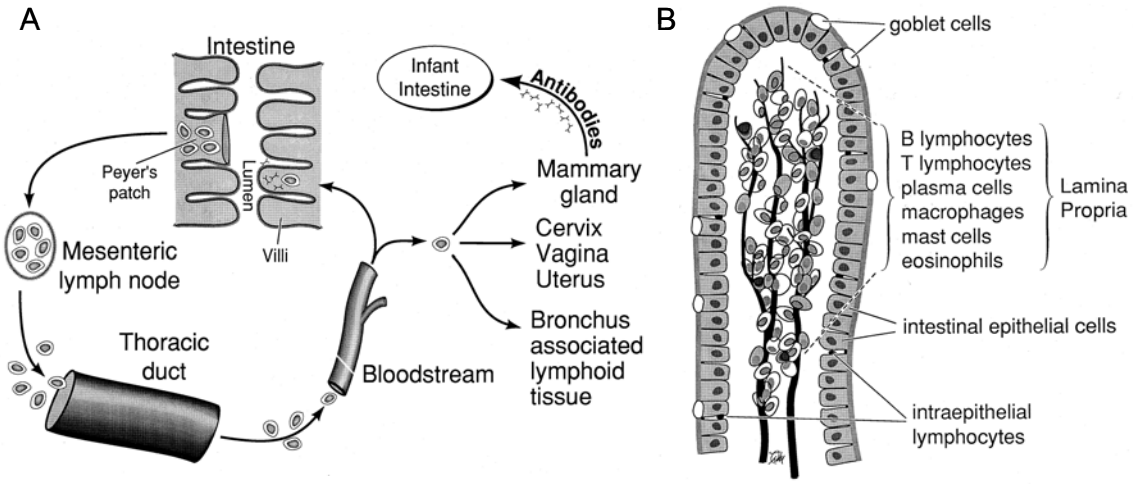


Figure 1. Gut-associated lymphoid tissue (A) and immune cells of the intestinal villus (B). Samples of the contents of the intestinal lumen are transported across the specialized epithelial cells covering the Peyer's patch and presented to lymphocytes (A). Activated lymphocytes leave the Peyer's patch and travel to the mesenteric lymph nodes where they continue differentiating and dividing. The lymphocytes eventually drain into the systemic circulation via the thoracic duct and home back to the intestinal mucosa and other mucosal tissues. Lymphocytes populate the lamina propria and intraepithelial spaces within the intestinal villi (B). Reprinted from Langkamp-Henken, B., Glezer, J.A., Kudsk, K.A.. Immunologic structure and function of the gastrointestinal tract. Nutr. Clin. Pract. 1992;7:100-108, with permission from the American Society for Parenteral and Enteral Nutrition (A.S.P.E.N.). A.S.P.E.N. does not endorse the use of this material in any form other than its entirety (28)

several other chemokine receptors were isolated within the lamina propria (24). Steinstad et al. (24) showed that CCR9 deficient cells transferred into recipient animals had reduced presence in the lamina propria. Johansson-Lindbom et al. (25) showed that mesenteric lymph node dendritic cells, but not splenic derived dendritic cells, generated α4β7, CCR9, $CD8^+$ T cells. Injection of this cell population into congenic mice showed that this T-cell population was localized to the small intestinal epithelium (25). A later study by the same investigators, found that the generation of $\alpha4\beta7^+$ $CCR9^+$ $CD8^+$ T cells which specifically homed to the GALT was dependent on dendritic cells bearing the $CD103^+$ marker (26).

Dendritic cells isolated from Peyer's patches, and not from spleen or peripheral lymph nodes, also have the ability to stimulate expression of gut homing α4β7 and CCR9 markers on $CD8^+$ T cells (27). The authors termed the ability of dendritic cells to mark the cells which they activate for later homing back to the specific tissue in which they were originally activated, "selective imprinting" (27). These data demonstrate the ability of gut-derived dendritic cells to influence the trafficking of lymphocytes back to their areas of activation through the use of chemokine receptors (which will be discussed in a later section). The return of activated lymphocytes to the GALT tissue in which they were activated allows for a more effective immune response against the pathogen that has invaded a specific area.

Cellular Populations in the GALT

The T and B lymphoid cells found in the GALT share similarities and differences with those found throughout the rest of the body. T-cell receptors (TCR) on either CD4 or CD8 positive T cells are typically heterodimers composed of an alpha and beta chain (TCRαβ) or of a gamma and delta chain (TCRγδ). In general, the dominant TCR in most of the body is TCRαβ. The TCRγδ T cell has less genetic diversity than TCRαβ T cells and are structurally similar to an immunoglobulin molecule (29).The TCRγδ only accounts for 3.8% of TCR in the peripheral blood of healthy adults (30), but their tissue distribution is specifically localized. TCRγδ represent as much as 43% of $CD45^+$ intraepithelial leukocytes (31). The Peyer's patches in the GALT have T and B cell rich zones, the T-cell zones are composed of approximately 65% $CD4^+$ TCRαβ and about 30% $CD8^+$ TCRαβ (32). The B cells in the germinal centers of the Peyer's

patches produce predominantly the antibody isotype IgA and some IgM, and therefore contain a high percentage of IgA positive B cells (32). Epithelial cells of the gut transport antibody across the cell via receptors. Antibody is then released into the gut lumen and a portion of the receptor remains attached to the antibodies providing stability within the lumen. This secretory component functions to keep the antibody in close proximity to the mucus layer and protects against antibody proteolysis by digestive enzymes, the IgA released into the lumen is therefore known as secretory IgA (sIgA). T-cell populations in mesenteric lymph nodes are mainly composed of TCRαβ$^+$ T lymphocytes with a small fraction containing the alterative T-cell receptor, TCRγδ. Like Peyer's patches and mesenteric lymph nodes, the population of TCRs in lamina propria is also similar in phenotypic distribution to the body, consisting of the previously mentioned CD4$^+$ and CD8$^+$ T-cell populations and B cells. Manzano et al. (33) report that CD45$^+$ leukocytes isolated from the lamina propria are 67% TCRαβ and 7.5% TCRγδ. Intraepithelial lymphocytes (IEL) residing between the epithelial cells (enterocytes) are mainly CD8$^+$, and comprise four main populations, all of which contain a CD8αα homodimer (34). Some cells expressing the CD8αα homodimer have been found to have an increased production of cytokines (35). Two populations include the CD4$^+$ and CD8αβ$^+$ TCRαβ T cells, similar to the more conventional T cells of the Peyer's patch, mesenteric lymph nodes, and lamina propria, except also bearing the CD8αα homodimer. TCRγδ T cells are present in high amounts in the mucosal epithelium (34, 2005 #71). This population of TCRγδ is CD4 negative and the

conventional CD8αβ heterodimer negative, but has a CD8αα homodimer instead (34). The final main population of T cells composing the intraepithelial lymphocyte population have the TCRαβ but are CD4 negative and the conventional CD8αβ heterodimer negative, but have a CD8αα homodimer (34). Manzano et al. (33) report the TCRαβ composing 45%, whereas the TCRγδ compose 43% of $CD45^{+}$ intraepithelial leukocytes in 7-week BALB/C mice. A difference between mice and humans is the lack of the TCRαβ that are CD4 and the conventional CD8αβ heterodimer negative, but have a CD8αα (in humans these are present in the human fetal period only) (34). TCRγδ cells, which compose a larger percentage of intraepithelial lymphocytes, appear to have a role in activation of apoptosis in damaged epithelial cells (35). Cellular populations found in GALT tissues are listed in Table 1. All of these cell populations and components of the GALT provide important immune functions that serve to create an effective barrier against potential pathogens.

Table 1-Normal GALT cellular populations

Author (reference)	Species/Strain	Gated Population	Tissue	CD3 (%)	CD8β (%)	CD8αα (%)	CD4 (%)	TCRαβ (%)	TCRγδ (%)
Fujihashi (36)	Mouse/ BALB/C	None (% total cells counted)	MLN	81.6					
Manzano (31)	Mouse/ BALB/C	$CD45^+$ Leukocytes	PP	34.7	3.3	7.6	36.1	43.4	0.7
			IEL	89.4	15.2	69.1	7.7	45.4	43.3
			LPL	77.9	17.6	37.1	36.3	66.9	7.5
Szczypka (37)	Mouse/ BALB/C	None (% total cells counted)	MLN	43.4	16.5		26.5		
Helgeland (38)	Rat	None (% total cells counted)	IEL	65.0	26.0	64	3	49	16
			MLN	55	8	9	47	54	1
Fujihashi (39)	Mouse/ C3H/HeN	CD3-None: Others presented as % of $CD3^+$	IEL	86.4	74.5		7.6		
Laky (40)	Mouse/ C57BL/6J X 129 Ola	$CD3^+$ cells	IEL						18-32

Abbreviations: mesenteric lymph nodes, MLN; Peyer's patches, PP; intraepithelial lymphocytes, IEL; lamina propria lymphocytes, LPL

T-Cell Signaling and the GALT

TCRαβ T cells bearing CD4 or CD8 require at least two signals for activation. The activation of T cells bearing the TCRαβ and TCRγδ have both similar and dissimilar characteristics. The primary signal for TCRαβ is the interaction between the CD4 or CD8 molecule/TCR complex and the antigen/major histocompatibility (MHC) complex on the antigen presenting cell. The antigen-specific TCRαβ^{+} T cells recognize that antigen in complex with the major histocompatibility protein and is stabilized by either the CD4 molecule (in the case of the MHC class II) or the CD8 molecule (for the MHC class I). The secondary signal for TCRαβ T cell is often an interaction between CD28 on the T cell and CD28 ligands [B7.1 (CD80) or B7.2 (CD86)] on the antigen presenting cell. CD28 is a constitutively expressed receptor on T cells, but can be upregulated at both mRNA and protein levels following TCR/CD3 stimulation (41), but decreases in aging (42). B7-CD28 and MHC-TCR activation of the TCRαβ^{+} T cell promotes IL-2 secretion and increases the cells responsiveness to IL-2. TCR-MHC complexes occur in lipid rafts formed within the T-cell membranes, which bring CD28, CD3, and CD4 or CD8 receptors in close proximity on the T-cell surface (43). The TCRαβ is associated with a complex of the proteins CD3 and CD4 or CD8, this resulting TCR complex has cytoplasmic regions that are associated with Src-family protein kinases. Upon receiving both TCR complex and CD28 signals and formation of the lipid raft, the Src-family protein kinases p59fyn (Fyn) and p56^{lck} (Lck) are activated by removal of inhibitory phosphate groups. The formation of the lipid raft brings the activated p56^{lck} associated with CD4 or CD8 in close proximity to it's downstream substrate

on the CD3 TCR, ZAP-70, beginning a signaling cascade via phosphorylation of ZAP-70 that culminates in the activation of transcription factors in the nucleus, such as NF-κB, initiating gene transcription, differentiation, and proliferation.

The signaling of the T cells bearing TCRγδ is less well characterized. Like TCRαβ T cells, data from Sperling et al. (44) demonstrated that TCRγδ T cells also need 2 signals for activation: the antigen-TCRγδ/CD3 complex and B7-CD28. The TCRγδ/CD3 complex does not require major histocompatibility proteins on antigen-presenting cells for recognition of antigen (45). Like their TCRαβ counterpart, CD28 was expressed at low levels until stimulation at which point surface expression of CD28 increased (44). Incubation of TCRγδ T cells with an antigen bearing cell line, with and without B7 showed that absence of CD28 costimulation prevented TCRγδ T-cell proliferation and IL-2 production (44). As for TCRαβ T cells, the addition of exogenous IL-2 to TCRγδ T cells and antigen bearing cell lines without B7 in vitro could replace CD28-B7 signaling as a costimulation (44). Sperling et al. (44) showed that a conventional $B7^+$ APC provided costimulation via CD28 to the TCRγδ T cell; however, nonconventional antigen presenting cells could activate TCRγδ T cells using BB-1, a different CD28 ligand or via antigen presentation with a non-traditional MHC molecule (46). Sperling et al. (44) proposed that the increased presence of TCRγδ T cells in the epithelial tissues may reflect their increased potential to be activated by nonconventional antigen presenting cells, such as epithelial cells. The differences obtained in potential signaling mechanisms and localization may be due to the presence of different subsets of TCRγδ T cells (47). Two main types of

TCRγδ T cells have been reported: Vδ1 and Vδ2 (47). TCRγδ T cell Vδ1 express CD57 and are $CD5^{-/lo}$, $CD28^{-}$, while TCRγδ T cell Vδ2 are $CD5^{hi}$ $CD28^{+}$ $CD57^{-}$ (47). TCRγδ T cell Vδ2, Vγ9 subpopulations are present in higher numbers in human peripheral blood (48,49), while TCRγδ T cell Vδ1 have been shown to be present in higher numbers in intestinal intraepithelial lymphocytes (30). The γδ TCR, like the αβ TCR, is associated with the CD3 protein complex, and forms clusters after signaling to trigger the downstream signaling cascade (29).

Arosa et al. (50) proposed that CD28 is an essential costimulatory molecule of T cells and stimulation of T cells in the absence of this costimulatory molecule can lead to T-cell anergy. CD28 costimulation is necessary for the formation of the lipid raft around the TCR complex site (51). Jordan et al. (52) proposed that the mechanism for this CD28 function was through cytoskeletal rearrangement. Tavano et al. (53) showed that a $CD4^{+}$ Jurkat T-cell line incubated with antigen stimulated antigen presenting cells, with and without B7 for cosignal engagement, resulted in Lck accumulation and localization at the lipid raft (immune synapse) only when the CD28 molecule was engaged. This phenomena was also seen in the peripheral blood lymphocytes of elders. $CD4^{+}$ T cells from elders demonstrated 5.5-fold decreased recruitment of Lck to lipid rafts compared to $CD4^{+}$ T cells from young individuals following stimulation and only a 34% association of CD28 in the lipid rafts from the cells from the elders versus 71% in the cells from the young (54). Larbi et al. (54) concurred with Tavano et al. (53) and concluded that ligation of the TCR and CD28 induced the recruitment of $p56^{lck}$ to the lipid raft. $CD4^{+}$ T cells stimulated in the absence of CD28

stimulation were unable to avoid clonal anergy as determined by lack of IL-2 production and cellular proliferation (55). Kundig et al. (56) showed that a transient signal at the TCR in CD28 deficient mice anergized $CD8^+$ T cells leading to a reduction in proliferation and cytotoxic activity. The cellular populations evaluated in the studies above did not specify whether the TCR was of αβ or γδ origin; however, the studies used peripheral $CD8^+$ or $CD4^+$ populations or $CD4^+$ Jurkat cell lines, so most likely these conclusions may be pertinent primarily to TCRαβ and select subsets of TCR γδ populations. Furthermore, two separate studies found that CD28 knockout mice failed to form germinal centers in their Peyer's patches (57) and $CD28^{-/-}$ mice had decreased numbers of Peyer's patches than heterozygous $CD28^{+/-}$ C57BL/6 mice (58).

Work by Arosa et al. (50) suggested the $CD8^+$ lymphocytes within the GALT are predominantly $CD28^-$. In this study, adult human peripheral blood $CD8^+$ $CD28^+$ lymphocytes cultured with colonic epithelial cells resulted in the expansion of the $CD8^+$ $CD28^-$ population (50). The authors hypothesized that the production of a $CD8^+$ $CD28^-$ intraepithelial T-cell population was the result of peripheral blood T cells that migrated to the epithelium and adhered to intestinal epithelial cells via gp180 or CD1d (50). The ability of intestinal epithelial cells to act as antigen presenting cells has been well documented by electron microscopy which showed soluble antigen taken up by endolysosomal pathways, expression of antigen with gp180 and CD1d (a non-classical MHC molecule), and engagement of the intestinal epithelial cell with the CD8 molecule (23). A complex is formed between gp180 and CD1d on the intestinal epithelial cell and

the CD8/TCR on the intraepithelial lymphocyte (50).The epithelial surface molecule gp180 interacts with T cells via CD8 to activate $p56^{lck}$ (50,59). Campbell et al. (59) proposed that this complex served as a non traditional class I MHC molecule which triggered regulatory rather than cytolytic T-cell functions. This theory was confirmed by studies showing monoclonal antibodies against gp180 blocked $CD8^+$ T-cell proliferation and the activation of the CD8 associated $p56^{lck}$ (23). Campbell et al. (59) suggested that IEC (intestinal epithelial cells) may play a role in the activation of a subset of T cells involved in the suppression of local mucosal, and systemic immune response. The idea of suppressive activity of the $CD8^+$ $CD28^-$ intraepithelial lymphocytes was also supported by the lack of the presence of the CD28 molecule. Work by Allez et al. (60) also supported this idea by showing that long-term-cell culture of human intestinal epithelial cells with human $CD8^+$ T cells caused expansion of the $CD8^+$ $CD28^-$ subpopulation. Cell and protein analysis by flow cytometry and mRNA expression showed that $CD8^+$ $CD28^+$ T cells stimulated by IL-2 for 6-8 weeks were almost entirely $CD28^-$ (61). The identification and characterization of this cell population provides essential insight into the mucosal cellular environment available to mount an immune response.

Cytokines and Chemokines

Cytokines play a crucial role in the orchestration of immune responses. These small proteins, typically weighing around 25 kDa, are released by immune and some non-immune cells in response to a stimulus and bind to receptor proteins on target cells. Cytokines alter the microenvironment and cell-to-cell communications through their ability to act in an autocrine, paracrine, or

endocrine manner, meaning that the cytokine released by a cell can influence itself, cells in the immediate vicinity, or distant cells (depending on the particular cytokine stability), respectively. Cytokines are divided into groups based on structural characteristics and functional properties. Pond et al. (62) showed that the profile of cytokines produced is stimuli-, mouse strain-, and tissue-specific, creating a great diversity in potential immunological responses. Cytokine gene expression in a primary infection differs between lymphocytes isolated from BALB/c mouse Peyer's patches, mesenteric lymph nodes, and splenocytes (63). When Svetic et al. (63) enterally challenged mice with a parasitic nematode, they saw no differences in cytokine expression in splenocytes, but significant alterations in Peyer's patch and mesenteric lymph node cytokine expression. Many studies have attempted to define the cytokine changes during zinc deficiency, measuring systemic and splenic cytokine patterns of expression and then using these findings to explain or make interpretations about the parasitic challenge results occurring within the intestinal tissue. However, studies by Svetic et al. (63) and Pond et al. (62) discredit this methodology, suggesting that due to tissue differences a more direct measurement of tissue cytokines is required to truly support such interpretations.

$CD4^+$ T cells are called T-helper (T_H) cells. There are two general subclasses of T_H cells: T_H1 and T_H2. Cytokine responses have been generally classified as either a T_H1 or a T_H2 response, based on the types of cytokines produced by these subsets of T-helper cells after antigen activation. T_H1 support

more inflammatory cell-mediated immune responses and a T_H2 response supports T-cell regulation of the humoral (antibody producing) cellular response.

Chemokines (7-12 kDa), a particular family of smaller cytokines which can be further subdivided based on amino acid structure, are also secreted products of T cells and are often the first proteins released by tissues in an immune response. Their primary role is to recruit leukocytes to the site of inflammation. Cytokines and chemokines work together to stimulate the endothelium of vessel walls to express selectins and integrins, slowing the flow of target cells passing in the bloodstream, aiding in the diapedesis of cells across the endothelium from the blood vessel into the tissue. Chemokines then provide a chemical gradient to allow for chemotaxis of the target cells to the area of inflammation. Chemokines and cytokines are an integral part of both the initial innate immune response and the adaptive immune response.

Cytokines, Chemokines and Their Importance in GALT

The essential role of cytokines and chemokines within the GALT, as in other tissues, is in the recruitment of leukocytes to sites of immune responses and inflammation. The mesenteric lymph nodes, Peyer's patches, and lamina propria contain high endothelial venules. Cytokines and chemokines act on endothelial cells lining the venules to promote the expression of selectins and integrins. These adhesion molecules allow for the adherence of circulating leukocytes to the walls of the high endothelial venules, followed by the diapedesis and chemotaxis of leukocytes into the tissues. In this way, cytokines and chemokines can recruit T cells and other populations essential for maintaining integrity of the mucosal barrier to the lamina propria and

intraepithelial spaces within the small intestine and the colon. The chemokines CCL 28 (chemokine ligand 28) and CCL25 are produced within the intestine by epithelial cells. These proteins bind to the receptors CCR10 (chemokine receptor 10) and CCR9, respectively, found on gut homing lymphocytes. Previous studies show that CCR9 deficient mice have a 50% reduction in IgA secreting plasma cells (64), alterations in the development of T and B cells, and a reduction in TCR$\gamma\delta^+$ IEL resulting in a decreased T cell to epithelial cell ratio (65). Furthermore, Hosoe et al. (66) showed that blocking of the CCL25-CCR9 pathway with an antibody against CCL25 inhibited the accumulation of LPL and IEL into the intestinal mucosa. Berin et al. (67) showed that human intestinal epithelial cells produced CCL22, resulting in the recruitment of $CCR4^+$ T cells, and the production of T_H2 anti-inflammatory cytokines. Human colon intestinal epithelial cells were also shown to express and secrete chemokines that could recruit many different cell types into the mucosal tissue (68). In this study, the stimulation of epithelial cells by bacteria upregulated mRNA and protein chemokine expression leading the authors to conclude that these chemokine changes may explain the differential appearance of leukocytes during a mucosal inflammatory response (68). Cytokines and chemokines play an important role as chemoattractants for cell populations in the maintenance of the mucosal barrier and in response to an inflammatory challenge.

Overall Immune Responses During Zinc Deficiency

As reviewed by Oteiza and Mackenzie there are numerous clinical situations that can lead to zinc deficiency, genetic conditions such as acrodermatitis enteropathica (a genetic condition, producing a severe zinc

deficiency), early parenteral nutrition, chronic diarrhea, malabsorption diseases, Crohn's disease, aging, and short bowel syndrome (69). Severe deficiency is relatively uncommon unless genetic conditions exist or in older studies evaluating parenteral nutrition; however, mild zinc deficiency can occur due to a number of the conditions listed above. Furthermore, zinc deficiency is associated with a characteristic decline in many parameters of immune function. Zinc deficiency has been linked to decreases in body weight, thymic weight, lymphocyte population, erythroid populations (red blood cell progenitors), natural killer cell activity, and delayed-type hypersensitivity; whereas zinc deficiency can simultaneously increase numbers of granulocytes and monocytes, and promote susceptibility to parasitic infections (14,16,70-75). Six-week-old A/J strain mice fed a zinc deficient diet (0.8 ppm) for 6 weeks showed a two-fold increase in plasma levels of the glucocorticoid corticosterone when compared to zinc-adequate mice (>50 ppm) (76). Glucocorticoids have been shown to cause death of immature thymocytes (77), increase circulating granulocytes numbers, and extend the life of neutrophils, all of which also occur during zinc deficiency (78,79). Zinc deficiency is also associated with increased oxidative stress, DNA fragmentation, altered transcription factor activity, such as NF-κB, ultimately affecting gene expression, and altered DNA repair mechanisms (71,72,80,81). Due to the importance of zinc structurally and functionally it has the potential to disrupt normal bodily functions at many different levels.

Previous studies have shown decreases in thymus weight and body weight as a result of zinc deficiency (70,82,83). Studies involving zinc-deficient models

are complicated by the fact that intake of a zinc-deficient diet is accompanied by a decrease in food intake; therefore, it is difficult to interpret which conclusions result directly from the zinc deficiency and which are the result of caloric restriction. This analysis problem necessitates the use of a pair-fed group, i.e. a group fed a zinc adequate diet but at the level of intake of the zinc deficient mice. In young A/J mice, 6 weeks of zinc deficiency (0.8 ppm) led to impaired weight gain in comparison to the zinc adequate group (>50 ppm), which continued to gain weight as expected according to their growth curve (76). King et al. (83) showed that using a 50-day zinc-deficient diet (0.5 to 0.6 ppm) to model a chronic-zinc deficiency in female A/J mice resulted in a 37% decrease in thymic weight, decreased body weight, but no signs of parakeratosis. The final mouse weights of the zinc-deficient mice in this chronic-zinc deficiency study were 74% of their zinc-adequate study mates that gained weight as would be expected by comparison to growth curves for this strain; however, the weights of the zinc-deficient mice were essentially identical to their baseline body weights. This implies that the zinc deficiency resulted in a lack of weight gain over the course of the 50-day study, but led to no weight loss in comparison to their baseline weights (83). The authors made comparisons between the zinc-adequate and zinc-deficient groups, excluding the pair-fed group, due to no differences seen in intake between the zinc-adequate and the pair-fed groups. These data are in contrast to earlier work by these investigators in which the female A/J mice fed a 0.8 ppm zinc-deficient diet experienced an 84% decrease in thymus weight and had body weight being 65% of the zinc-adequate group and less than their

baseline weight by day 24 of this severe zinc deficiency (16). The pair-fed group in this study had 88% of the body weight of the zinc-adequate mice (16). Furthermore, the weight loss in these severely zinc deficient mice was accompanied by a high degree of parakeratosis (16). Although the later study showed only impairment of weight gain while the earlier study showed weight loss in zinc deficiency, the authors did not explain the differences in the weight data obtained between the two studies. One possible explanation for the reduction in thymus weights could be attributed to increased apoptosis due to the elevated corticosterone levels that occur in zinc deficiency, as previously mentioned. Removing the source for the glucocorticoid by adrenalectomy of zinc deficient mice (0.8 ppm) resulted in thymus weights equivalent to zinc-adequate mice (>50 ppm) (76). These adrenalectomized, zinc-deficient mice with normal thymus weights did have alterations in their thymus composition after four weeks on the diet (76); zinc-adequate mice had a normal 2:1 (cortical:medullary) ratio, while the zinc-deficient mice had a 1:1 ratio (76). The authors hypothesized that the ability to maintain thymus weight but alter compartment size in zinc deficiency may be due to the redistribution of thymocytes (76). Although there are discrepancies on the exact differences that occur in thymus and body weight due to zinc deficiency, most authors concur that there are some negative consequences on growth and the thymus as a result of zinc deficiency.

Zinc Deficiency and Gene Expression

Zinc deficiency has been shown to alter gene expression in vivo and in vitro. Cousins et al. (84) found that cDNA microarray and quantitative PCR of RNA from human mononuclear cells (THP-1) cultured under zinc-supplemented

and zinc-deficient conditions showed approximately 5% of the 22,216 genes expression were zinc-responsive. When the zinc responsive genes were divided into subgroups based on functional characteristics, genes involved in signal transduction, immune/cytokine function (cytokines & receptors), nucleic acid binding, metabolism, apoptosis, cell growth/development, and cytoskeleton were identified as being zinc responsive (84). Some of these changes in gene expression, but not all, may be attributed to metal-responsive transcription factor 1 (MTF-1), a transcription factor containing 6 zinc fingers (85). MTF-1, in the presence of zinc, binds to metal responsive elements in certain genes and induces gene transcription. Metallothionein is an example of such a MTF-1 dependent, zinc-responsive gene (86). Zinc deficiency in a non-differentiated T-cell line resulted in decreased binding of the zinc-finger transcription factor NF-κB to DNA (13). Analysis of the effects of zinc deficiency on gene expression in rat liver via cDNA and oligonucleotide arrays showed the presence of zinc-responsive genes with 66 out of 1550 observable genes altered in zinc deficiency (87). Studies by Moore et al. (71,72) identified zinc-dependent changes in gene expression in thymocytes from CD-1 mice fed a 3-week zinc-deficient (<1 ppm) versus zinc-adequate diet. Genes important in T-cell development and activation, such as $p56^{lck}$, heat-shock proteins, MHC class II molecules, and a T-cell cytokine receptor, were altered by zinc deficiency (71,72). One study in rats using differential mRNA display on mRNA from rat intestinal tissue identified 13 zinc-regulated genes important in signaling, growth, and transcription, with a 1.5

fold difference (88). Collectively, these data suggest a tissue dependent effect of zinc deficiency in the regulation of gene expression.

Zinc Deficiency and Cellular Changes

Many changes in the immune function that occur during zinc deficiency may be due to tissue specific changes in lymphocyte populations. Absolute numbers of nucleated cells obtained from the bone marrow of moderately and severely zinc-deficient 6-week-old A/J mice were were not different than mice fed a zinc-adequate (28 ppm) or a pair-fed diet; however, the composition of the subpopulations was altered by zinc deficiency (as discussed below) (70). The moderate and severely zinc deficient mice were distinguished by subdividing the group as follows: after receiving a zinc-deficient diet (<1 ppm) for 34 days, a mouse was considered moderately zinc deficient if the weight was 73-75% of the zinc-adequate group, and severely zinc deficient if the mouse weight was 68-71% of the zinc-adequate group (70). In this study, the erythroid cell population decreased approximately 25% and 60% in the moderately and severely zinc-deficient groups, respectively. The erythroid cell population composed 18.5% of the nucleated cells extracted from the bone marrow in the pair-fed and zinc-adequate group, but only 13.7% in the moderate, and 9.1% in the severe zinc-deficiency groups (70). The authors suggest that losses in the erythroid cell compartment may explain the anemia that often occurs in zinc deficiency (70). The lymphoid subpopulation of the bone marrow, which gives rise to both T and B lymphocytes, decreased by 50% and 70% in moderately and severely zinc-deficient mice, respectively, in comparison to pair-fed and zinc-adequate mice (70). Evaluation of the pre-B-cell lineage within the bone marrow found pre-B cell

losses were only 15% in moderately zinc-deficient mice, versus 75% for severely zinc-deficient mice (70). The granulocyte population, which is composed of neutrophils, basophils, and eosinophils, increased by 36% in the moderately zinc-deficient mice and 57% in the severely zinc-deficient mice bone marrow (70). Granulocytes, as a percentage of the nucleated cell population, represented 40%, 54%, and 62%, in the zinc-adequate, moderately zinc-deficient, and severely zinc-deficient diet groups, respectively (70). Bone marrow monocyte populations were 70% larger than in zinc-adequate mice in both the moderately and severely zinc-deficient diet groups (70). The decreases in percentages and overall numbers of erythrocytes and lymphocytes were balanced by increases in the percentages and overall numbers in the granulocyte and monocyte populations, resulting in no change in the overall nucleated cell populations in the bone marrow during zinc deficiency. King and Fraker hypothesize that downregulation of the metabolically draining lymphopoiesis may allow the body to conserve nutrients in the face of micronutrient deficiency (70). A later study by the same investigators evaluated a 50-day feeding cycle to mimic a chronic-zinc deficiency (83). Evaluation of the nucleated cell populations from the bone marrow identified a 35% decrease in the erythroid cell population and no other changes in other subpopulations (83). If zinc deficiency leads to increases in neutrophils and monocytes, often the first cells to arrive to the location of in inflammatory challenge, they may enable the innate immune system to compensate for the lymphocyte alterations that have the potential to disrupt the adaptive immune response.

The effects of zinc deficiency on T-cell populations in peripheral blood have also been evaluated in rats (17). Peripheral blood from 3-week-old Sprague Dawley rats fed a zinc-deficient diet (<1 ppm), zinc-adequate diet (level not stated in paper), or pair-fed diet for 3 weeks was analyzed for the presence and proportions of T-cell subpopulations. No differences in T-cell subpopulations were seen except that fewer $CD90^+$ TCRαβ T cells were found in the blood of zinc-deficient growing rats than in the pair-fed and zinc-adequate groups (17). T cells that have recently undergone maturation in the thymus express the protein CD90 (17). The peripheral T-cell lymphopenia that occurs in zinc deficiency is linked to pre-T cell losses via apoptosis. Thymic atrophy also plays a role in the lymphopenia that occurs in zinc deficiency due to inability to replete the dwindling T-cell populations. Furthermore, subpopulation alterations that occur in zinc deficiency may shift the T-cell subpopulations selected within the thymus and then released into the periphery.

Evaluation of thymocytes following zinc deficiency has resulted in conflicting data. These differences may relate to the interpretation of the data by the investigators and how the data were presented. In two separate studies on 3-week-old rats, Hosea et al. (17,89) observed decreased thymus weights in zinc-deficient (<1 ppm), compared to pair-fed or zinc-adequate (30 ppm) groups following 3-weeks of diet. However, if thymus weights were expressed as a percentage of body weight, no changes were detectable (17,89). In both of these studies the author failed to identify differences in the absolute number of lymphocytes isolated per gram of thymus weight (17,89). Two additional studies

using 4-6 week-old A/J mice fed a zinc-deficient (0.5 ppm) or a zinc-adequate (28 ppm) evaluated over a time-course of 31 days and longer, found that thymus weights in the zinc deficient mice were 38% (after 31 days of diet), 58% (after 45 days), and 63% (after 50 days) of that of the zinc-adequate mice (15,83). These mice had corresponding decreases in thymocytes extracted (49-80%) in comparison to zinc-adequate mice (15,83). Although these data suggest that decreases in thymus weight are associated with decreased cellularity, and that lack of weight change of thymuses results in lack of overall changes in cell numbers, the earlier studies did not report overall thymocyte numbers only the normalized (number/gram of organ weight) were reported. Thymocytes from 6-week-old A/J mice fed a 3-week zinc-deficient diet (0.5 ppm) resulted in a three-fold increase in apoptosis in $CD4^+$ $CD8^+$ pre-T cells versus zinc-adequate mice, resulting in a 40% decrease in $CD4^+$ $CD8^+$ pre-T cells in the thymus of zinc-deficient mice (15). Mature T cells ($CD4^+$ $CD8^-$ and $CD4^-$ $CD8^+$) had no increases in apoptosis as a result of the zinc deficiency (15). A 50-day chronic-zinc deficiency (in 4-week-old A/J mice, fed 0.5 ppm) resulted in a 60% increase in apoptosis of pre-T cells (83). Thymocytes from zinc-deficient rats (<1 ppm diet for 3 weeks) had increased $CD4^-$ $CD8^+$ TCRαβ percentages compared to zinc-adequate diet-fed rats (17). A separate study showed that 3 weeks using similar diets with rats resulted in decreased (35-52%) thymocyte pre-T cells ($TCR\alpha\beta^-$ $CD4^+$ $CD8^+$ and $TCR\alpha\beta^+$ $CD4^+$ $CD8^+$) in both zinc deficient (<1 ppm) and pair-fed rats in comparison to zinc adequate (30 ppm), in addition thymocytes from zinc-deficient rats had lower $TCR\alpha\beta^+$ $CD4^+$ $CD8^-$ (T helper) populations versus zinc-

adequate rats (89). The pre-T-cell lymphopenia may be caused by increased glucocortocoid levels in zinc deficiency (mentioned above), which may lead to thymic atrophy as a result of apoptosis that occurs in zinc deficiency (83,90). The involution of the thymus that occurs during zinc deficiency may also be associated with decreased ability to distinguish self from non-self and a decrease in the number of mature T cells (although the data are inconclusive).

The effect of zinc-deficiency on the spleen was evaluated. Hosea et al. (89) found that 3-week-old rats fed a zinc-deficient diet (<1 ppm) for 3 weeks had a 20% decrease in spleen weight in comparison to baseline values, while pair-feeding resulted in no change, and spleen weights from zinc-adequate fed mice increased 78% over baseline. However, normalization of the spleen weight to body weight resulted in no differences among groups (89). In a separate study by the same authors, rat spleen weights were significantly lower ($P<0.05$, 336 mg ± 23, mean ± SEM) in zinc-deficient mice (<1 ppm, 3-week feeding period) than pair-fed (405 ± 24) or zinc-adequate mice (743 ± 37); however, normalization by body weight resulted in no difference among groups (0.23% to 0.25%, spleen weight/body weight) (17). Moderately and severely zinc deficient A/J mice, weighing 66% and 72% of the zinc-adequate group, respectively, both had an approximate 45% decrease in splenic lymphocytes (91). In this study, the severely zinc-deficient group also showed parakeratosis (91). Six-week-old A/J mice defined as severely zinc deficient, weighing only 65% of the zinc-adequate mice and having parakeratosis following 31 days of a 0.8 ppm diet, exhibited a 26% increase in T-helper cells, no change in cytotoxic T cells, and a 20%

increase in the T-helper/T-cytotoxic ratio in comparison to a zinc-adequate group (16). This same study identified only small decreases (5 and 8%) in the B-cell population ($B220^+$) in moderately, weighing 70-72% of zinc-adequate mice, and severely zinc-deficient mice, respectively (16). This B-cell population decrease was attributed to the non-IgM and non-IgD, immunoglobulin positive subset (16). Rats fed a zinc-deficient (<1 ppm) diet for 3 weeks showed no changes in splenocyte T-cell subpopulations in comparison to pair-fed and zinc-adequate mice (17). This study identified fewer $CD90^+$ TCRαβ T cells in splenocytes isolated from the zinc-deficient rats versus the pair-fed and zinc-adequate rats (17). These data are in contrast to a later study by the same authors which found that splenocytes from 3-week-old rats fed a zinc-deficient diet (<1ppm) for 3 weeks had 40% to 63% fewer T-helper cells ($TCR\alpha\beta^+$ $CD4^+$ $CD8^-$) and fewer T-cytotoxic cells ($TCR\alpha\beta^+$ $CD4^-$ $CD8^+$) versus the pair-fed and zinc-adequate diet groups (89). Furthermore, spleen weight and T-cell populations recovered within 7 days during zinc repletion in comparison to 23 days for thymic weights and subpopulations (89). The spleen is an important lymphocyte-rich organ which assists in the ability to fight infections. Hence, alterations in splenic functional capacity during zinc deficiency could have negative impact on immune responses.

Although lymphopoiesis appears to be affected during zinc deficiency (70,92), the remaining cells have normal immune responses (91). Adult mice with decreased zinc intake show losses of pre-B and pre-T cells and associations of these losses with decreases in protective Bcl-2 levels, suggesting another

possible mechanism for altering lymphopoiesis (4). The protein Bcl-2 is present on the outer mitochondrial membrane, and increased BCL-2 levels have been shown to be protective against apoptotic mechanisms (93).

The function of the residual lymphocytes remaining after zinc deficiency has been shown to be comparable to pair-fed and zinc adequate mice, therefore, the overall decreased immune response due to zinc deficiency can most likely be attributed to the overall decreases in lymphocyte numbers described above or potentially due to shifts in cell subtypes (91,94). Cook-Mills et al. (91) compared the T-lymphocyte functionality after a zinc-deficient diet was fed for 30 days. Splenocytes were isolated and cultured for evaluation of proliferative responses and IL-2 production in response to stimulation with a mitogen (concanavalin A), and in a mixed lymphocyte culture system (using mitomycin C-treated C57BL/6 target cells). Culturing in vitro allows for normalizing the differences in cell numbers obtained from zinc-deficient, pair-fed, and zinc-adequate mice by performing each assay with an equal number of cells. Lymphocyte proliferative responses and IL-2 production in response to concanavalin A were similar among the zinc-deficient, pair-fed, and zinc-adequate mice (91). In mixed lymphocyte culture the zinc-deficient splenocytes had a higher proliferative response and IL-2 production than those from zinc-adequate mice (91). This may be related to the exposure of splenocytes to elongated steroid exposure which has been shown to increase mixed lymphocyte culture responses (95,96). These data are supported by the work of Dowd et al. (94) in which splenocytes isolated from rats fed a zinc-deficient or a pair-fed diet for 4 weeks had equivalent

proliferative responses to concanavalin A and IL-2 production. The in vivo cell culture experiments of Cook-Mills et al. (91) and Dowd et al. (94) created a zinc-deficient culture environment by using autologous serum from zinc-deficient animals in the zinc-deficient cell cultures. Investigators in an earlier study had shown decreases in extracted splenocyte T-cell responses, but in vitro cultures of these splenocytes contained fetal-bovine serum and not autologous serum from zinc-deficient animals (97). Splenocytes were removed from zinc-deficient, zinc-adequate, or pair-fed mice injected 5 days earlier with sheep red blood cells (SRBC), and the function of B cells was analyzed by their ability to form anti-SRBC antibodies, IgM anti-SRBC, and IgG anti-SRBC (91). Although the number of antibody producing cells was lower per spleen, the amount of antibody per antibody-producing cell was equivalent between the zinc-deficient, pair-fed and zinc-adequate mice (91). The lymphopenia that occurs in zinc deficiency may result in immune dysfunction; however, the remaining lymphocytes appear to retain their functional characteristics.

No studies have evaluated the changes in cellular populations present in the gastrointestinal tissue as a result of zinc deficiency. The changes in immune function at the level of the gastrointestinal tract during zinc deficiency may be the indirect result of changes to systemic immune system development that are perpetuated into the GALT.

Zinc Deficiency and Cytokine Responses

Zinc deficiency is associated with an imbalance between T_H1 and T_H2 cytokines (13,14,98). Two studies evaluating the effect of a mild zinc deficiency on human peripheral blood mononuclear cells which were isolated and then

stimulated in vitro, resulted in a decline in the T_H1 cytokines IL-2 (at 8,12, and 20 weeks) and IFN-γ (at 20 weeks) (99,100). In one of these studies there was no effect of zinc deficiency on the T_H2 cytokines IL-4, IL-6, and IL-10 at 20 weeks (100). Analysis of cytokine production by HuT-78 cells, a human malignant T lymphoblastoid cell line, cultured in zinc-deficient culture conditions showed 40% less IL-2 protein and a 50% decrease in IL-2 mRNA following stimulation (13). BALB/c mice fed a zinc-deficient (<1 ppm) diet for four weeks were unable to respond to a nematode (parasitic) challenge, which requires a T_H2 cytokine-mediated immune response, resulting in an increased presence of worms (101). Splenocytes from these mice stimulated in culture with the parasite antigen had decreased IL-4, IL-5, and IFN-γ in the infected group in comparison to the zinc-adequate mice, and no detectable IL-4 in the uninfected zinc-deficient mice (101). The same authors, found that purified T cells from splenocytes of BALB/c mice exposed to the same zinc-deficient conditions as in the previous study had decreased IL-4 and IL-5 cytokine production in comparison to pair-fed and zinc-adequate mice (102). Furthermore, splenocyte populations from both the zinc-deficient and pair-fed mice had decreased IFN-γ production from T cells and decreased IL-4, IL-5, and IFN-γ production by antigen presenting cells in comparison to zinc-adequate mice (102). This suggests that food restriction rather than zinc restriction produced these changes. These authors also evaluated a primary versus secondary immune response in BALB/c mice exposed to a 4-week zinc-deficient (<1 ppm), pair-fed, or zinc adequate (60 ppm) diet given a parasitic challenge, which was treated, and then the mice were re-

exposed to the same parasitic challenge (103). Stimulation in culture with parasitic antigen resulted in decreased IL-4 levels in zinc-deficient mice following primary infection but not secondary infection, and decreased IFN-γ following secondary challenge but not after primary challenge. Bao et al. (98) evaluated cytokine expression from several different cell lines following 24 days of culture in zinc deficient conditions. They found that zinc deficiency altered both cytokine mRNA and protein concentrations. Decreases were identified in IL-2 and IFN-γ, while increases were found in IL-1β, IL-8, and TNF-α. The authors concluded that zinc deficiency affected cytokine patterns at the level of gene expression, and that differences in expression were dependent of the cell lineage. The effect of zinc deficiency on T_H1 or T_H2 cytokine expression may be dependent on the experimental system evaluated (human versus mouse), type of challenge (primary versus secondary, or parasitic versus bacterial), and the level of zinc deficiency. The impaired ability of zinc-deficient humans or mice to produce T_H1 and T_H2 cytokines would result in an increased susceptibility to immunological challenges, as was seen in the parasitic studies described above.

Zinc Deficiency and Gut Associated Lymphoid Tissue (GALT)

In addition to the inability of zinc deficient mice to protect their intestinal tract against parasitic infections as mentioned above, many studies link zinc deficiency with increased diarrhea in mice and humans suggesting disturbance of the gut barrier (104-106). The gut is an important mediator in zinc homeostasis (107). The body has no “long-term storehouse” of zinc (107). However, epithelial cells in the intestinal mucosal system contain transport and trafficking proteins capable of facilitating or limiting zinc translocation from the lumen into the

bloodstream. Fraker and King as recently as February of 2004 noted “how little we know of the impact of zinc deficiency on mucosal immunity” (4).

The imbalance between T_H1 and T_H2 cytokines may be one mechanism by which zinc deficiency promotes disturbances of the gut mucosa. Individuals with zinc deficiency are more susceptible to intestinal parasitic infections which require T_H2 responses for effective elimination from the gut associated lymphoid tissue (GALT) (4). “Many studies have documented the early synthesis, production, and uptake of T_H2 cytokines by GALT” as important for the production of effective immune responses (108). As previously described, although Prasad et al. (13) reports only T_H1 cytokine differences, Scott et al. (14) showed zinc deficiency decreased T_H2 cytokine expression and responses in intestinal tissue following parasitic challenge. Interestingly, parasitic infections are associated with increased IgE production and transport into the lumen of the intestine (63,109). The ability of T cells with TCRγδ origin (which are present in unusually high concentration in the mucosa) to signal the B cells to switch isotype production to IgE is dependent on the presence of T_H2 cytokines (108). This is supported by the work of Shi et al. (103) who showed that BALB/c mice fed a zinc-deficient diet (<1 ppm) for four weeks and exposed to a parasitic challenge had decreased IgE production in comparison to pair-fed or zinc-adequate mice, but no differences were identified among groups for IgG. The failure of zinc-deficient mice to maintain their T_H1 and T_H2 cytokine production can inhibit the ability of these mice to protect their gut via IgE antibody production. Furthermore, the intestinal epithelia is one of the most rapidly turning

over tissues in the body and has the potential to be affected by zinc deficiency which is associated with increased apoptosis as previously mentioned (110).

It is essential that the intestine is able to limit the responsiveness to antigens that are frequently encountered in our food to prevent hypersensitivity reactions within the gut. This resulting tolerance is T-cell specific and mediated by the induction of T_H2 cytokines and suppression of the T_H1 inflammatory cytokines (20). This induction of antigen tolerance also involves regulatory T cells (T_{reg}) and the production of a major suppressive cytokine, TGF-β. Since T_H2 cytokines are known to decrease during zinc deficiency, it has been hypothesized that the ability to induce tolerance in zinc deficient animals would be impaired (111). Finamore et al. (111) evaluated the sensitivity of the intestine to food antigens during zinc deficiency. Repeated exposure in vivo to ovalbumin failed to induce tolerance in zinc-deficient mice as measured by stimulation of mesenteric lymphocytes and splenocytes with ovalbumin in vitro compared to pair-fed controls, and a resulting decrease in T_H2 cytokines (111). However, the 28 days it took to induce tolerance was at a point where many studies have shown adverse intestinal changes as a result of zinc deficiency. The failure to evoke tolerance may have been due to a concurrent inflammatory response, which was found to exist in this study based on neutrophil infiltration and histology (111). The authors concluded that the inability for zinc-deficient mice to develop tolerance as a result of cytokine changes would lead to a more easily inflamed intestinal mucosa (111). These changes at the cellular level have the capacity to interfere with gut homeostasis.

Studies show that zinc-deficient animals have increased iNOS expression, and the shift from T_H2 to T_H1 cytokines may be responsible for this iNOS increase. PCR showed iNOS mRNA levels in the small intestine increased in zinc-deficient mice versus pair-fed mice (106). Immunohistochemistry of the intestinal segments following addition of cytokines known to invoke an acute-phase response, showed an increase in anti-iNOS staining in the zinc-deficient group (106). This staining was localized to the basal layer and dispersed throughout the villus cells (106). Furthermore, addition of the iNOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME) to the drinking water of mice fed the zinc-deficient diet decreased iNOS mRNA expression and apoptotic cells in the intestinal villi versus mice fed the zinc-deficient diet without L-NAME (105). Nitric oxide (NO), the product of the iNOS enzyme, leads to tissue damage when produced in excess. Canali et al. (112) showed that addition of polyphenols, an antioxidant in red wine, to a zinc-deficient diet decreased the number of macrophages and neutrophils that migrated into the intestinal mucosa and decreased the production of inflammatory cytokines. These data again show the association of iNOS through its production of NO with intestinal damage in a zinc-deficient animal model. Another mechanism for the diarrhea that occurs in zinc deficiency is increased uroguanylin (113). Levels of preprouroguanylin mRNA were 2.5-fold more abundant in rat intestine during zinc deficiency (113). Uroguanylin, a natriuretic peptide hormone, binds guanylate cyclase C (which is also bound by the Escherichia coli enterotoxin) and alters fluid balance in the intestine (113). The diarrhea that is associated with zinc deficiency in many

cases not only exasperates the zinc deficiency itself but leads decreased absorption of other nutrients.

Zinc and T-Cell Signaling

T-cell signaling in activation involves the interaction of many components, several of which have been shown to be altered in zinc deficiency. These include IL-2, IL-2 receptor (IL-2R), and $p56^{lck}$. IL-2 is essential in the activation of T cells, which can then release IL-2 to act in an autocrine and paracrine function to stimulate proliferation. In vitro stimulation following zinc-deficient culturing of a T lymphoblastic cell line showed decreased IL-2 production (98). Furthermore, mildly zinc-deficient human subjects have also been shown to have lower IL-2 production from T_H1 lymphocytes (74). Prasad et al. (13) found that the HuT-78 cell line cultured in zinc-deficient conditions produced 50% less IL-2 mRNA, and had a 70% decrease in the soluble IL-2 receptor (sIL-2R) in comparison with zinc-sufficient cells. These changes in IL-2 and sIL-2R data were confirmed at the protein level via western blotting (13). IL-2 and/or IL-2R deficient mice were shown to have an inability to develop self-tolerance, and increased incidence of inflammatory bowel disease (114-116).

An increase in $p56^{lck}$, a zinc-finger protein with an essential role in the perpetuation of a cell surface stimulation signal to the intracellular cascading signaling events, has been shown to increase in zinc deficiency (72,117,118). Lepage et al. (117) showed that splenocytes isolated from C57BL/6 mice fed a zinc-deficient diet (<1 ppm) for 4 weeks had increased $p56^{lck}$ protein as visualized via western immunoblotting. Cells isolated from the pancreas of CD-1 mice fed a zinc-deficient diet (<1 ppm) for 3 weeks had increased $p56^{lck}$ as

measured by cDNA array (>1.5-fold increase), RT-PCR, and western analysis (>80% increase) in comparison to mice fed a zinc-adequate diet (72). Lin et al. (119) showed the binding of $p56^{lck}$ to glutathione S-transferase (GST) which is complexed with the cytosolic portion of CD4 or CD8 is dependent on the zinc. Moore et al. (72) hypothesized that metallothionein may act as a donor and/or acceptor for zinc in the zinc-dependent $p56^{lck}$ interaction with CD4 or CD8, and that disturbance of the interaction between CD4/CD8 and $p56^{lck}$ may trigger the cell to upregulate $p56^{lck}$ expression due to a feedback mechanism. Overexpression of $p56^{lck}$ has been shown to alter T-cell maturation (decreasing $CD4^+$ $CD8^+$ generation from $CD4^-$ $CD8^-$ precursor cells) (120), increase the incidence of thymic tumors (121), and decrease the presence of TCRs on the cell surface by 75% due to increased lysosomal degradation (122). Sohn et al. (123) found that transgenic mice overexpressing $p56^{lck}$ also had decreased double positive $CD4^+$ $CD8^+$ thymocytes, but had an increased presence of single positive $CD4^+$ and/or $CD8^+$ thymocytes; however, increased $p56^{lck}$ led to decreased survival of peripheral T cells. Alterations of $p56^{lck}$ may provide one mechanism through which zinc deficiency is able to alter T-cell maturation, signaling, and phenotype charactersitics.

Other molecules altered by zinc deficiency that may affect T-cell signaling include TNF-α and the MHC class II molecule. Bao et al. (98) showed that zinc deficiency in cell culture increased TNF-α concentrations. This alteration in TNF-α can be attributed to a zinc finger protein, tristetrapolin (TTP), capable of being modulated by zinc concentrations in cell culture (124), which decreases the half-

life of TNF-α mRNA (125). Peritoneal macrophages from TTP deficient mice overproduced TNF-α (126). Furthemore, in vitro culture of a T-cell line with TNF-α for 8 weeks resulted in the specific decreased expression of the T-cell costimulatory molecule CD28 without altering other T-cell markers (CD4 or CD3) (127). These data provide a potential mechanism by which zinc deficiency may induce CD28 costimulatory marker changes, although no studies have evaluated the direct relationship between zinc deficiency and CD28. The mRNA of the Class II MHC molecule, which presents antigen to the TCR/CD3 complex on $CD4^+$ T cells, was shown to decrease in thymocytes from CD-1 mice fed a zinc deficient (<1 ppm) diet for 3 weeks (71). Decreased presence of class II MHC molecule, a necessary antigen presenting molecule throughout the body, in the thymus may alter positive and negative selection during the maturation process. Moore et al. (71) concluded that reduction in the MHC Class II receptor contributes “to the lymphopenia of zinc deficiency or the pathogen-specific increased susceptibility to infectious disease seen secondary to a zinc deficiency”; in addition, alterations in any of the signal or costimulatory molecules that may occur during zinc deficiency could lead to these negative consequences.

Purpose of this Work

Despite protective mechanisms, increased susceptibility to parasitic infection and inflammation occur within the gut especially during zinc deficiency. Changes in cellular populations as a result of zinc deficiency are well documented systemically; however, differences in their tissue distribution exist based on the differential expression of tissue-specific chemokines and cytokines

as shown by Pond et al. (62). The purpose of this study is to determine if the systemic cellular lymphopenia and cytokine changes that have been shown to occur are perpetuated into the GALT as mice become progressively deficient in zinc. If these changes occur within the short-lived epithelial tissues, this would explain the inability of zinc-deficient animals to develop tolerance (111). Furthermore, zinc-deficiency induced weakening and thinning of the epithelium could lead to increased uptake and presentation of molecules as antigenic, resulting in the potential for activation and functional changes in T-cell subpopulations in the GALT. Changes in lymphocyte populations were evaluated in mice fed one of three diets, either a zinc-deficient, zinc-adequate, or a pair-fed diet for 9 weeks. Cells were isolated from the Peyers patches, mesenteric lymph nodes, and intraepithelial lymphocytes and lamina propria lymphocytes of the small intestine, and intraepithelial lymphocytes from the colon, for flow cytometric analysis of T-cell subpopulations present. Based on systemic losses of naïve cell populations previously observed during zinc deficiency (4), it would be expected that changes in the subpopulations would occur. As a first look at T-cell functional activation, the effects of a 9-week progressive zinc deficiency on expression of the signaling molecule CD28 on T cells was evaluated in all tissues. Tissue from the colon, the site of the symptomatic effects of zinc deficiency, was assessed for global changes in cytokine and chemokines at the transcript level. Evaluation of these changes within the GALT during zinc deficiency could provide insight into the increase in gastrointestinal illnesses that

occur during zinc deficiency which are likely the result of a breakdown in the mucosal barrier.

MATERIALS AND METHODS

Animals

Specific pathogen free (SPF) BALB/c mice (4-week-old, Harlan) were obtained, acclimated in microisolater cages for 7 days, and then housed individually in acid-washed, hanging stainless steel cages on a 12-h light/dark cycle with free access to distilled, deionized water. Entrance into the mouse facility required gowning and personal protective equipment as in SPF facilities, although our housing facilities were not SPF. Mice were fed an AIN-76A-based pelleted diet formulated with egg white protein, containing 30 mg zinc/kg of diet (Research Diets, Appendix A) for the first week, 6 mice were killed to obtain baseline values, and the remaining mice were randomly assigned to the same zinc-adequate diet (ZA, n=11/group) or a zinc-deficient diet (<1 mg zinc/kg diet, ZD, n=11/group). A group was pair-fed (n=11/group) the ZA diet to the level of the ZD mice, but was later dropped from the analysis because differences in weight between the ZA and ZD diet groups were not observed (Appendix B). At various time points over the 9 weeks mice were anesthetized with halothane and killed by exsanguination via cardiac puncture and cervical dislocation in accordance with the University of Florida Institutional Animal Care and Use Committee approved protocol (#D963). The blood was collected, allowed to clot for 30 min at room temperature, centrifuged at 1850 x g for 15 min, and serum was removed and stored at -20°C for measurement of serum zinc. Mouse thymic

and liver tissues were removed and weighed. The small intestines and colons were removed and flushed with 10 and 5 mL, respectively, of 1 X Hank's Buffered Saline Solution (HBSS, without Ca^{2+} or Mg^{2+}, Fisher Scientific) and placed in HBSS/HEPES [15 mM HEPES (Fisher Scientific) in HBSS without sodium bicarbonate, pH 7.2] on ice. Small intestinal tissue was processed for intraepithelial lymphocytes (IEL), lamina propria lymphocytes (LPL), and Peyer's patch lymphocytes. Mesenteric lymph nodes (MLN) were removed for isolation of lymphocytes. Colonic tissue was processed for the isolation of colon intraepithelial lymphocytes (cIEL only, not LPL). A 5 mm section of the colonic tissue used to isolate RNA was placed into 10 volumes of RNAlater (Ambion, Inc.) at 4^{o}C for 24 hrs, after which the supernatant was removed and the tissue was stored at -80°C.

Determination of Serum Zinc Concentrations

Five-fold dilutions of serum in dH_20 were analyzed for zinc content by atomic absorption spectrophotometry using an AAnalyst 100 Atomic Absorption Spectrophotometer (Perkin Elmer) and compared to a 0.2-1.0 ppm standard curve.

Lymphocyte Extraction from Intestinal Tissue

The procedure for isolation of lymphocytes from tissues was developed based on modifications and combinations of previously described methods (40,66,128). Peyer's patches and MLN were processed separately but similarly. MLN and Peyer's patches were removed from the small intestines, fat was removed, and tissues were cut into small sections and cells were extracted by gentle application of pressure using a 5-mL syringe plunger. Peyer's patch tissue

homogenate was resuspended in 20 mL of 50 U/mL type I collagenase in RPMI complete [10% fetal bovine serum-heat inactivated (FBS, Mediatech), 15 mM HEPES, 0.1 mg/mL gentamyacin, in RPMI-1640 containing L-glutamine but without sodium bicarbonate, pH 7.2, all reagents were obtained from Fisher Scientific], incubated at 37^{o}C for 1 hr on a rocker. MLN homogenate was resuspended with 20 mL RPMI-complete. MLN and Peyer's patches tissue suspensions were then propelled through a 100 um mesh. Extracted lymphocytes from each tissue were washed twice with RPMI complete (the first Peyer's Patch wash was in 50 mL; all other washes were in 20 mL) at 450 x g for 10 min, resuspended for counting in 1.0 mL of FACS buffer [1% bovine serum albumin, 0.1% sodium azide, 1 X phosphate buffered saline (PBS), pH7.4], and stained with fluorescent conjugated antibodies and then analyzed as described below in the Flow Cytometry section.

The remaining small intestinal and colonic (with Peyer's patches removed) tissues had fat removed, were cut lengthwise, and then were cut trans-sectionally into 5-mm sections and placed into a petri dish containing 10 mL of HBSS/HEPES (1 X HBSS, 15 mM HEPES, pH 7.2). The contents of the Petri dish were poured over a 250-um nylon-mesh screen held taunt by an embroidery hoop and suspended over a petri dish. These tissue pieces were returned to a petri dish rinsed with 10 mL of HBSS/HEPES, and again filtered by pouring over the same 250-um mesh apparatus. This procedure was repeated until the tissue had been washed 6 times with HBSS/HEPES and filtered through the 250-μm nylon mesh each time. The remaining tissue pieces were then transferred into a

siliconized flask containing 10 mL of 37°C HBSS/FBS/EDTA (10% FBS, 15 mM HEPES, 5 mM EDTA, 0.1 mg/mL gentamycin, in HBSS, pH 7.2). This was stirred at 100 rpm (Fisher Electronic Stirrer Model 2008) for 15 min at room temperature. The contents of the flask were filtered through a fresh 250-µm nylon mesh filter (as described above), the tissue pieces were returned to the flask, a fresh 10 mL of room temperature HBSS/FBS/EDTA was added, and the flask was again stirred at 100 rpm for 15 min at room temperature. This procedure was repeated until the tissue pieces had been washed and stirred 4 times. The filtrate from these washings was retained, pooled, and stored on ice for processing of IEL (the tissue was processed for LPL as described below). The IEL containing filtrate was centrifuged at 400 X g for 5 min and resuspended in 4°C RPMI-1640. A nylon wool column was prepared by the teasing and packing of 0.3 g of pre-washed nylon wool into a 10-mL disposable column. This column was equipped with a 23-gauge needle attached to a 3-way stopcock. The column was pre-wetted with 20 mL of 4°C RPMI-1640, and the column was not allowed to run dry. The resuspended cells were applied to the column, allowed to run completely through the column, and 10 mL of 4°C RPMI-1640 was added for elution. The entire 15 mL of flowthrough was centrifuged at 400 X g for 10 min, the pellet was resuspended in 0.5 mL of FACS buffer and stained with antibody conjugates for flow cytometric analysis (described in the Flow Cytometry section below). The remaining tissue pieces from the small intestine were processed for LPL (LPL were not isolated from the colonic tissue). The tissue pieces for LPL processing were washed for 5 min in 20 mL of RPMI complete by stirring at 100

rpm at room temperature. This wash was filtered through a 250-μm nylon mesh (described above) and the tissue retentate was digested by incubation at 37^{o}C for 1 hr with 20 mL of 100 U/mL type VIII collagenase in RPMI complete. The products of this incubation were filtered through a 250-μm nylon mesh and the filtrate was centrifuged at 850 X g for 10 min, washed twice in 20 mL of CMF/HEPES with centrifugation at 850 x g for 10 min, resuspended in 0.5 mL of FACS buffer, and stained for Flow Cytometric analysis as described in the following section.

Flow Cytometry

Cell marker expression of T-cell subpopulations were evaluated using flow cytometry in LPL, IEL, cIEL, MLN, and small intestinal Peyer's patch cell populations at various time points over the 9-week zinc deficiency protocol. Cell suspensions of 1.0 X 10^6 cells in FACS buffer containing 2 ug of unlabeled mouse IgG (Southern Biotech), for Fc receptor and non-specific antibody binding block, were prepared from each lymphocyte population. For each of the cell populations isolated and treated with mouse IgG, a separate tube was prepared for determination of background fluorescence for each antibody by addition of an appropriate fluorochrome conjugated to an isotype control (BD Biosciences Pharmingen). The following antibody conjugates (1 μg/1 X 10^6 cells) were added to stain the cells: fluorescein isothiocyanate (FITC) conjugated anti-TCRγδ, allophycocyanin (APC)-anti-CD3, phycoerythrin (PE)-anti-CD28, FITC-anti-CD4 and biotinylated-anti-CD8β (BD Biosciences Pharmingen). The anti-CD8β-labelled cells were subsequently detected by steptavidin-peridinin chlorophyll-a protein (SAv-PERCP) secondary antibody. In addition to the isotype control

tubes, the isolated cell suspensions containing mouse IgG were used as follows: to one tube antibodies were added for the evaluation of TCRγδ, CD8β, CD3, and CD28, and a separate tube was prepared with CD4, CD8β, CD3, and CD28 antibodies. After a 15-min room-temperature incubation, 200 ul of 4% formaldehyde in PBS was added. Samples were incubated for 10 min at room temperature and cells were washed twice with 1 mL of FACS buffer and centrifuged at 600 x g for 5 min each time. Cells were resuspended in 0.4 mL of FACS buffer for analysis. Isotype controls were used to set the crosshairs to define the negative populations, and then analysis of the forward scatter versus the CD3 identified the $CD3^+$ population. Flow cytometric measurements were performed by counting 10,000 $CD3^+$ cells using a FACSCaliber (BD Biosciences). Data were analyzed as the percent of total cells and as a percent of the $CD3^+$ population using FCS Express version 3.0 (De Novo Software).

Isolation of RNA

All experiments were done using RNAse inhibitor spray (Continental Lab Products) and DEPC treated water to minimize RNAse contamination. RNA was isolated from colonic tissue samples (approximately 16.5 mg, stored as previously described) from mice exposed to 3 and 9 weeks of a ZA or ZD diet, n=1/week/diet group, by homogenization and isolation according to the manufacturer's directions using the RNeasy kit (QIAGEN Inc.) for use in Microarray. RNA was quantitated using a Nanodrop Spectrophotometer (Nanodrop Technologies) and 4 ug of RNA was diluted 1:1 v/v with glyoxal sample loading dye (Ambion, Inc.) containing ethidium bromide, and treated according to manufacturers directions. Samples were loaded on a 1% (in TAE)

agarose gel and electrophoresis was performed at 100 V. Ethidium bromide intercalation into the RNA was visualized to check quality using a UV transilluminator.

RNA from remaining colonic tissue samples in mice fed for 9 weeks was extracted for use in quantitative real-time PCR (q RT-PCR, 20 mg each, n=4/diet group) using a modification of the Chomczynski RNA isolation method (129). Tissue was homogenized on ice in 1.0 mL of RNA-Bee (Tel-Test) using a TissueTearor (Fisher Scientific) on a setting of 3 for 30 seconds. The sample was centrifuged for 10 min at 10,000 x g at 4^{o}C. The supernatant was removed, 0.2 mL of chloroform was added, and samples were incubated on ice for 5 min. The suspension was centrifuged at 12,000 x g for 15 min at 4^{o}C. The top aqueous phase (0.45 mL) was removed and an equal volume (0.45 mL) of isopropanol was added. This was mixed well, incubated at room temperature for 10 min, centrifuged for 15 min at 12,000 x g at 4^{o}C, and the supernatant was removed. The pellet was washed with 1.5 mL of 75% ethanol and centrifuged for 10 min at 8,000 x g at 4^{o}C. The supernatant was poured off and the pellet was allowed to air dry for several minutes. The pellet was reconstituted with water and RNA was quantitated at 260 nm using a Beckman DU640 Spectrophotometer (Beckman Coulter, Inc.). RNA concentration was determined using the OD_{260} X 0.04 μg/μL(because 1 Absorbance Unit at 260 nm=40 ug/mL) X dilution factor =μg RNA/μL. All RNA to be used for q RT-PCR was DNase treated according to the manufacturer's directions with TURBO DNase (Ambion, Inc.) before analysis. Absorbance at 260 nm was measured using a Nanodrop Spectrophotometer to

quantitate nucleic acid content of the extracted and DNase treated RNA for use in q RT-PCR.

Microarray

RNA prepared as described above from weeks 3 and 9 in ZA and ZD mice for microarray analysis was the template for preparation of cRNA using the TruLabeling-Amp 2.0 kit (SuperArray Bioscience Corp.). Briefly, cDNA was prepared from 1.8-6 ug of RNA and used as the template for the subsequent cRNA synthesis reaction. The cRNA synthesis reaction involved the addition of biotinylated UTP. The biotinylated cRNA was purified using the SuperArray ArrayGrade cRNA cleanup kit (Superarray Bioscience) according to the manufacturer's recommendations and quantitated on a Nanodrop Spectrophotometer as above. The biotinylated cRNA was used for a pathway-specific Oligo GE Array Mouse Inflammatory Cytokines and Receptors Microarray according to manufacturer's recommendations (Superarray Bioscience Corp., complete gene listing in Appendix C). Briefly, after hybridization of 1.5 ug of biotin-labeled cRNA to the microarray, it was washed, blocked to reduce background staining, conjugated with alkaline phosphatase-conjugated streptavidin, and the image was captured with X-ray film following addition of chemiluminescent substrate. Data were analyzed with GEArray Expression Analysis Suite (GEASuite) Online Image Data Acquisition and Analysis Software (SuperArray Bioscience, version 1.1, Feb. 26, 2005)

Quantitative Real-Time PCR (q RT-PCR)

DNase treated RNA (500 ng) prepared as described above from the ZA and ZD mice was used as a template for the preparation of cDNA using the iScript

cDNA Synthesis kit (Bio-Rad Laboratories, Inc.). The iScript cDNA Synthesis kit uses random hexamer and oligo dT primers for the synthesis of cDNA from all RNA transcripts by the enzyme reverse transcriptase. Ten-fold serial dilutions of cDNA simultaneously prepared from samples known to contain the gene of interest functioned as standards in the q RT-PCR. cDNA samples were diluted 1:50 and used for q RT-PCR performed using cDNAs plus gene-specific primers and iQ SYBR Green Supermix detection system (Bio-Rad). Primers were designed using Primer Express Software (version 2.0, Applied Biosystems) and purchased from MWG Biotech. In constructing the primers, the entire sequence for the gene of interest was obtained from GenBank database (available through NCBI), the glycine (G) and cytosine (C) content were limited to 30% (with G/C's at the 5' end), the forward and reverse primers were matched for optimum temperatures, and length was a minimum of 19 and maximum of 22 nucleotides. The forward and reverse primer sequences obtained were blasted through the GenBank database to ensure that the primary matches obtained were the genes of interest. Primer sequences designed for chemokine ligand 25 (CCL25), chemokine ligand 17 (CCL17 also known as TARC), chemokine ligand 8 (CCL8 also known as MCP-2), interleukin 18 (IL-18), interleukin 1β (IL-1β), and 18S rRNA are listed in Table 2. A melt curve was done for each primer set prior to its use for q RT-PCR. Primers for metallothionein (MT) are described elsewhere (130). The dilutions of cDNA samples were compared to threshold cycles obtained from the 10-fold serial dilutions of standard cDNA curves during the

exponential phase of product synthesis. Fluorescence generated from the SYBR Green dye intercalation and primer melt curves were measured using a

Table 2. Q-RT PCR primer sequences

Gene	Orientation	Sequence
Chemokine ligand 25 (CCL25)	Forward Reverse	5′-ccaccaacgtcccagcatgt-3′ 5′-ggtgagtgggagggccttta-3′
Chemokine ligand 17 (CCL17 or TARC)	Forward Reverse	5’-gagctggtataagacctcagtggag-3’ 5’-tggccttcttcacatgtttgtc-3’
Chemokine ligand 8 (CCL8 or MCP-2))	Forward Reverse	5’-tgctttcatgtactaaagctgaaga-3’ 5’-ctacacagagagacataccctgctt-3’
Interleukin 18 (IL-18)	Forward Reverse	5’-gaaccccagaccagactgataata-3’ 5’-cttgttcttacaggagagggtagaca-3’
Interleukin 1β (IL-1β)	Forward Reverse	5’-tgggcctcaaaggaaagaatc-3’ 5’-ggtattgcttgggatccacact-3’
18S rRNA	Forward Reverse	5’-cgaggaattcccagtaagtgc-3’ 5’-ccatccaatcggtagtagcg-3’

Bio-Rad iCycler with the following amplification sequence: 2 min at 50^{o}C, 10 min at 95^{o}C, followed by 40 cycles of 15 seconds at 95^{o}C and 1 min at 60^{o}C. Data were analyzed using iCycler iQ Optical System Software version 3.0a (Bio-Rad Laboratories, Inc.). The threshold for detection was set 10 times greater than the baseline fluorescence signal. A duplicate set of samples and standards were similarly and simultaneously plated for enumeration of 18S rRNA levels. Expression levels of all genes were normalized to 18S rRNA transcript levels.

Statistical Analysis

Differences between ZA and ZD groups were analyzed using an unpaired two-tailed Student’s t-test analysis when variances were equal. When variances were unequal, data were analyzed using a nonparametric two-tailed t test. Standardized (ZD/ZA) cell phenotype data was analyzed using a one-sample t test with a hypothetical mean=1. Cell subpopulations were correlated with

chemokine mRNA transcript levels using the Pearson correlation. Results are expressed as mean ± SEM.

RESULTS

Mouse Weights

All mice gained weight over the 9-week feeding protocol. There was no difference between the percent of baseline weight in the ZD versus the ZA mice at weeks 3, 6, or 9 of the feeding period (Fig. 2). Additionally, ZD mice showed no outward signs of zinc deficiency, such as parakeratosis or diarrhea.

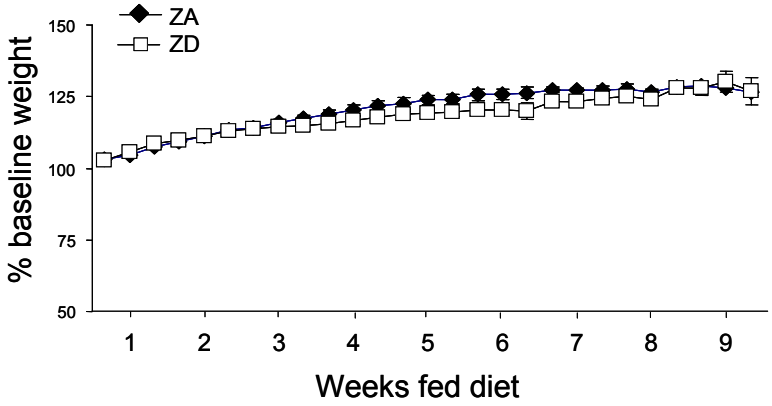


Figure 2. Percent baseline weight of BALB/c mice fed a zinc-adequate (ZA) or zinc-deficient (ZD) diet. Values are expressed as mean ± SEM. Baseline values represent 6 mice, ZA had n=11/group, and ZD had n=11/group. Numbers decreased over time as mice were killed at various time points over the 9 weeks, ending with an n=4 in each group. There was no difference between the percent of baseline weight in the ZD versus the ZA mice at week 3, 6, or 9 of the feeding period.

Organ Weights

Livers and thymuses were removed from ZA and ZD mice and weighed. No differences were identified in mean weight at any time point for either the livers or thymuses from the ZA or ZD mice.

Zinc Status Assessment

Serum zinc concentrations decreased progressively over the 9-week feeding protocol in the ZD versus the ZA mice (Fig. 3A). Serum zinc was significantly lower in ZD mice (P<0.01) at 6 and 9 weeks than ZA mice. Relative colonic MT mRNA gene expression was determined by normalization with 18S rRNA. Relative colonic MT mRNA levels of ZD mice were more than 3-fold lower than ZA mice (Fig. 3B, P<0.05).

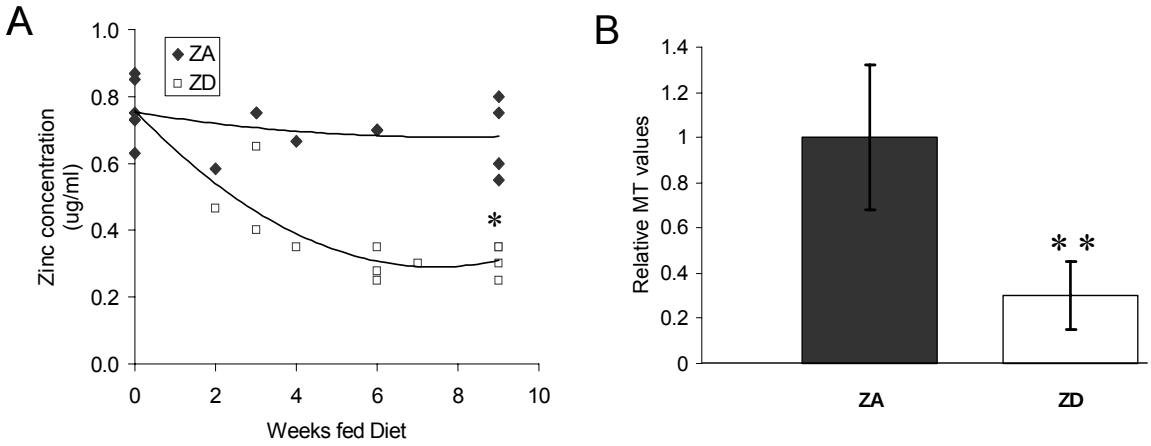


Figure. 3. Serum zinc concentrations and colonic metallothionein mRNA levels. Serum zinc concentrations over 9 weeks (A, n=6 at baseline, n=11 ZA, n=11 ZD) and relative colon metallothionein (MT) mRNA gene expression at week 9 (B, n=4/diet group) in BALB/c mice fed a zinc-adequate (ZA) or zinc-deficient (ZD) diet. Zinc concentrations were measured by atomic absorption spectrophotometry, and each point (Fig. 2A) represents a different mouse. Serum zinc was significantly lower (*P<0.01, ZD vs. ZA) at 6 and 9 weeks in mice fed the ZD diet. Colon MT expression was evaluated with q RT-PCR and normalized to 18s rRNA. MT values were significantly lower with ZD (**P<0.05, ZD vs. ZA). Values (Fig. 2B) represent the relative mean ± SEM.

Flow Cytometry

Overall cells numbers obtained and lymphocyte marker expression were evaluated in cellular populations isolated from all tissues (MLN, small

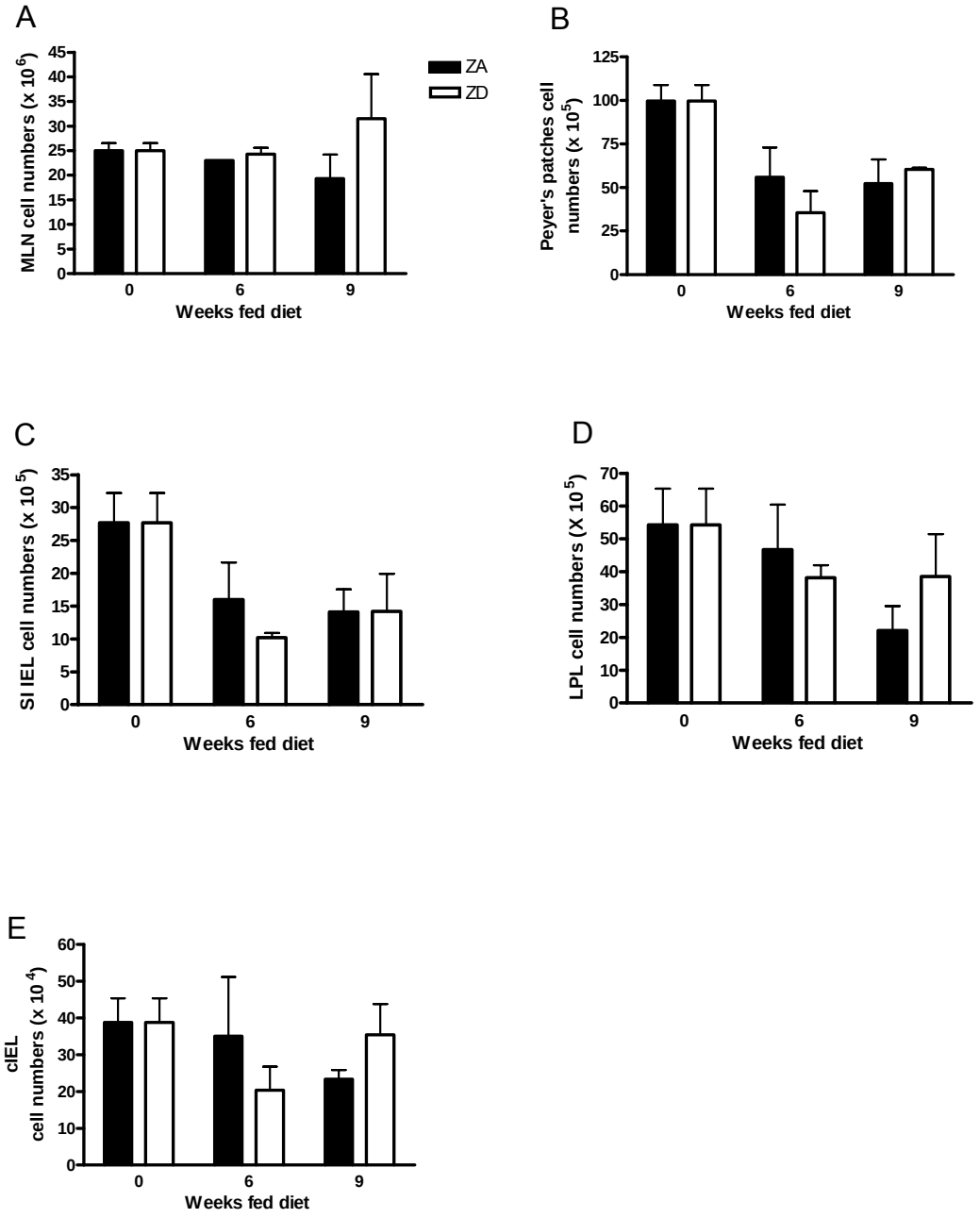


Figure 4. Total recovered cell numbers in GALT. Total cells recovered from mesenteric lymph nodes (A), Peyer's patches (B), and small intestinal intraepithelial lymphocytes (SI IEL, C), lamina propria lymphocytes (LPL, D), and colon intraepithelial lymphocytes (cIEL, E) in BALB/c mice fed a zinc-adequate (ZA) or zinc-deficient (ZD) diet. There was no affect of diet on overall number of cells recovered from the various tissues. There was a significant week effect in Peyer's patch and small intestinal intraepithelial lymphocytes (P<0.05). Data represent the mean ± SEM, n=2 to 6 mice per diet/week.

intestinal Peyer's patches, IEL, and LPL, and cIEL) during the 9-week zinc deficiency.

The total number of cells extracted from each tissue was determined for GALTs from ZA and ZD mice at weeks 0, 6, and 9. There was no effect of diet on total number of cells extracted from the mesenteric lymph nodes (Fig. 4A), Peyer's patches (Fig. 4B), IEL (Fig. 4C), LPL (Fig. 4D), and cIEL (Fig. 4E). There was a significant week effect, with total number of cells extracted from Peyer's patches and IEL lymphocytes decreasing with fewer cells in later weeks in comparison to week 0 (baseline), P=0.001 and P=0.03, in Peyer's patch lymphocytes and IEL, respectively.

Relative $CD3^+$ levels (ZD/ZA) as a percent of total cells increased with the progressive zinc deficiency in cIEL (Fig. 5, P=0.059, R^2=0.54). cIEL $CD3^+$ cells as a percent of total cells was greater at week 9 in ZD versus ZA mice (42.8 ± 3.9 versus. 29.1 ± 2.5, respectively, P=0.04). The percentage of total $CD3^+$ cells when analyzed independently of other cell markers was not significantly different in the other GALT examined, although differences were identified for several $CD3^+$ subpopulations in these tissues and are described below.

The $CD3^+$ subpopulation $TCR\gamma\delta^-$ $CD8\beta^+$ was altered by zinc deficiency in Peyer's patches and cIEL. The percentage of these $CD3^+$ $TCR\gamma\delta^-$ $CD8\beta^+$ lymphocytes was greater at all time points (ZD/ZA>1.0, P<0.01) in cIEL, but lower at all time points (ZD/ZA<1.0) in Peyer's patches lymphocytes in ZD relative to ZA mice as a percentage of total extracted cells (Fig. 6, P<0.01). Alterations in other $CD8\beta^+$ subpopulations at week 9 of the zinc deficiency

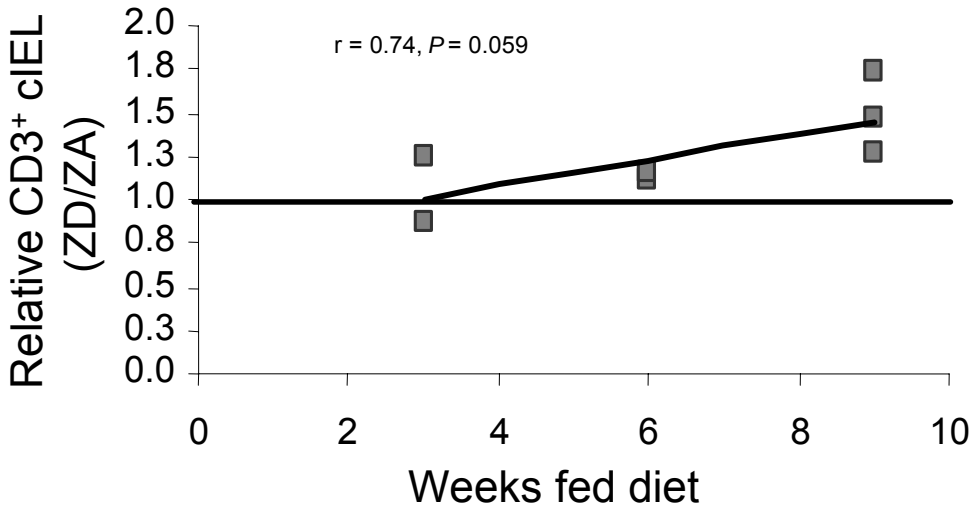


Figure 5. Colonic $CD3^+$ intraepithelial lymphocytes (cIEL) in BALB/c mice. cIEL $CD3^+$ mice fed a zinc-deficient diet (ZD) relative to mice fed a zinc-adequate diet (ZA). The percentage of $CD3^+$ cIEL increased in the colon with progressive zinc deficiency. n=2 at week 3 and 6, n=3 at week 9. Ratios based on percentages of total cell population.

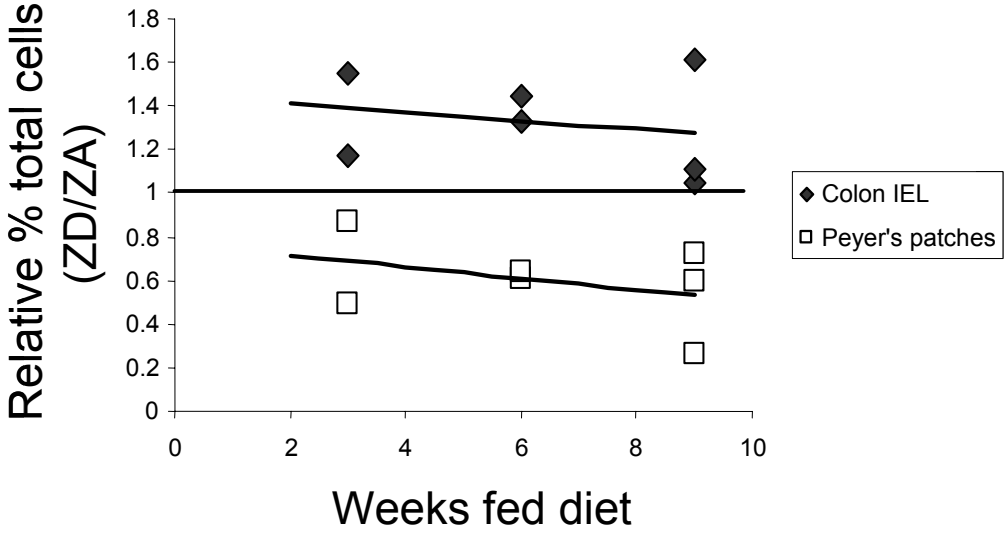


Figure 6. Colonic intraepithelial and small intestinal $CD3^+$ $TCR\gamma\delta^-$ $CD8\beta^+$ Peyer's patches lymphocytes in BALB/c mice. $CD3^+$ $TCR\gamma\delta^-$ $CD8\beta^+$ colonic intraepithelial and small intestinal Peyer's patches lymphocytes in mice fed a zinc-deficient diet (ZD) relative to control mice fed a zinc-adequate diet (ZA). The percentage of $CD3^+$ $TCR\gamma\delta^-$ $CD8\beta^+$ cells was higher in colonic IEL (ZD/ZA >1, n=7, $P<0.01$) but lower in Peyer's patch lymphocytes (ZD/ZA <1, n=7, $P<0.01$). Ratios based on percentages of total cell population.

protocol were also identified in Peyer's patches and MLN as either a percentage of total cells or as a percentage of $CD3^+$ cells and are listed in Table 3. No changes in $CD8\beta^+$ populations were identified at week 9 for the small intestine IEL or LPL populations.

$CD3^+$ $TCR\gamma\delta^+$ $CD8\beta^-$ lymphocyte subpopulations in Peyer's patches were significantly elevated as a percentage of total cells (Fig. 7, $P<0.05$), and as a percentage of $CD3^+$ cells (9.6 ± 0.6 versus 4.0 ± 0.5, ZD and ZA, respectively, $P=0.002$) in the ZD versus the ZA mice at week 9. Changes were not observed in the $CD3^+$ $TCR\gamma\delta^+$ T-cell subpopulations, for the phenotypic markers specified above, at week 9 in the other GALT examined.

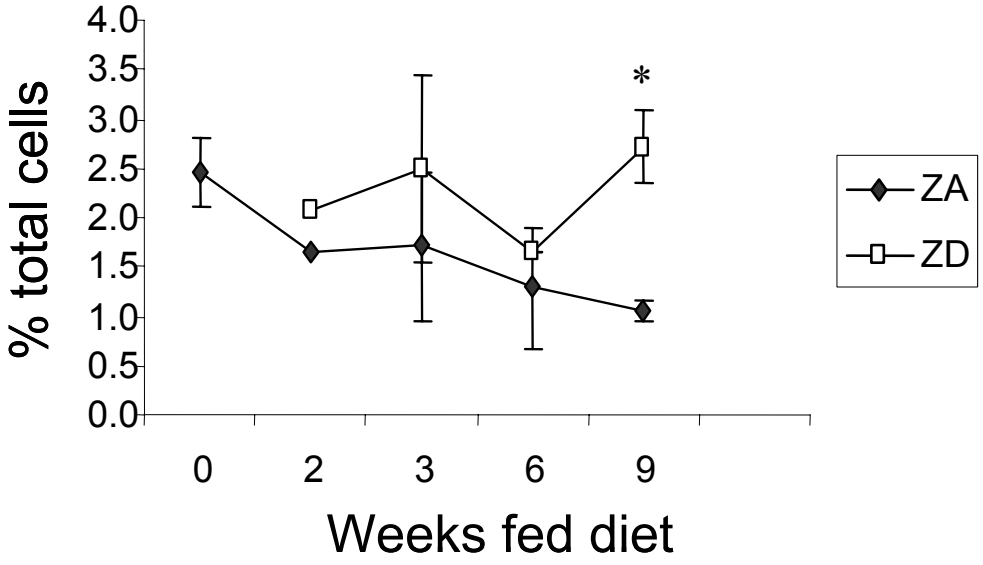


Figure 7. Small intestinal Peyer's patch $CD3^+$ $TCR\gamma\delta^+$ $CD8\beta^-$. Small intestinal Peyer's patch $CD3^+$ $TCR\gamma\delta^+$ $CD8\beta^-$ lymphocytes as a percentage of the total recovered cell population in BALB/c mice fed a zinc-adequate (ZA) or zinc-deficient (ZD) diet. *$P<0.05$, ZD vs. ZA at week 9. Values represent mean ± SEM, n=6, 1, 2, 2, and 3/group at week 0, 2, 3, 6, and 9, respectively.

$CD4^+$ T-cell subpopulations also had observed changes. Lymphocytes isolated from Peyer's patches in ZD mice had lower $CD4^+$ $CD8\beta^+$ populations as

a percentage of $CD3^+$ cells versus ZA mice at week 9 (Table 3). cIEL $CD4^+$ lymphocytes as a percentage of $CD3^+$ cells were lower in ZD versus ZA mice at 9 weeks (12.2 ± 1.8 versus 18.9 ± 0.5, respectively, *P*=0.02). Changes were not observed in these $CD4^+$ subpopulations at week 9 in the other GALT examined.

Table 3. $CD8\beta^+$ and $CD4^+$ populations in various tissues after 9 weeks of zinc-adequate and zinc-deficient diet.

Phenotype	Tissue	Gate	ZA diet	ZD diet	*P* value
$CD3^+$ $CD8\beta^+$	MLN	NG	20.8 ± 0.7	18.4 ± 0.4	0.04
$CD3^+$ $CD4^-$ $CD8\beta^+$	PP	NG	2.8 ± 0.3	1.5 ± 0.1	0.01
$TCR\gamma\delta^-$ $CD8\beta^+$	PP	$CD3^+$	11.6 ± 2.0	5.2 ± 0.6	0.04
$CD4^+$ $CD8\beta^+$	PP	$CD3^+$	19.6 ± 0.1	14.3 ± 0.8	0.003
$CD4^-$ $CD8\beta^+$	PP	$CD3^+$	10.6 ± 0.6	5.64 ± 0.5	0.003
$CD4^+$	cIEL	$CD3^+$	18.9 ± 0.5	12.2 ± 1.8	0.02
$CD4^+$ $CD8\beta^-$	cIEL	$CD3^+$	8.5 ± 1.7	3.7 ± 0.8	0.06

Abbreviations: not gated, NG (% of total cells); colon intraepithelial lymphocytes, cIEL; mesenteric lymph nodes, MLN; Peyer's patches, PP

Changes were observed in T-cell subpopulations containing the costimulatory marker $CD28^+$. Peyer's patches isolated from ZD mice had lower $CD28^+$ $CD4^-$ $CD8\beta^+$ populations as a percentage of $CD3^+$ cells (14.0 ± 2.4 versus 22.9 ± 2.0, ZD and ZA, respectively, *P*=0.048). $CD4^+$ $CD28^+$ cell populations were lower in ZD mice as a percentage of $CD3^+$ cells (4.4 ± 0.5 and 10.4 ± 0.2, ZD and ZA, respectively, *P*=0.0004) in cIEL. Further confirmation of these data was obtained when the $CD3^+$ $CD4^+$ $CD28^+$ cell population was evaluated as a percentage of total cells, this population was also lower in ZD mice as a percentage of total cells (1.8 ± 0.04 versus 3.1 ± 0.3, ZD versus ZA, respectively, *P*=0.014). Changes were observed for three $CD28^+$ cell populations isolated from the mesenteric lymph nodes. The MLN $CD3^+$ $CD28^+$ cell population was lower at week 9 in ZD versus ZA mice as a percentage of total cells (Fig. 8, *P*=0.03) or $CD3^+$ cells (39.6 ± 2.8 versus 62.1 ± 7.1, ZD versus ZA, respectively, *P*=0.04).

The MLN TCRγδ$^+$ CD8β$^-$ CD28$^+$ CD3$^+$ population was lower at week 9 in ZD mice as a percentage of total cells (0.1 ± 0.1 versus 0.9 ± 0.2, ZD versus ZA, respectively, *P*=0.017) and as a percentage of CD3$^+$ cells (13.4 ± 10.9 versus 57.0 ± 14.5, ZD versus ZA, *P*=0.074). The MLN TCRγδ$^-$ CD8β$^+$ CD28$^+$ CD3$^+$ cell population was higher at week 9 in ZD mice as a percentage of total cells (1.1 ± 0.1 versus 0.6 ± 0.1, ZD versus ZA, respectively, *P*=0.02) and as a percentage of CD3$^+$ cells (6.1 ± 0.4 versus 3.3 ± 0.6, ZD versus ZA, respectively, *P*=0.02).

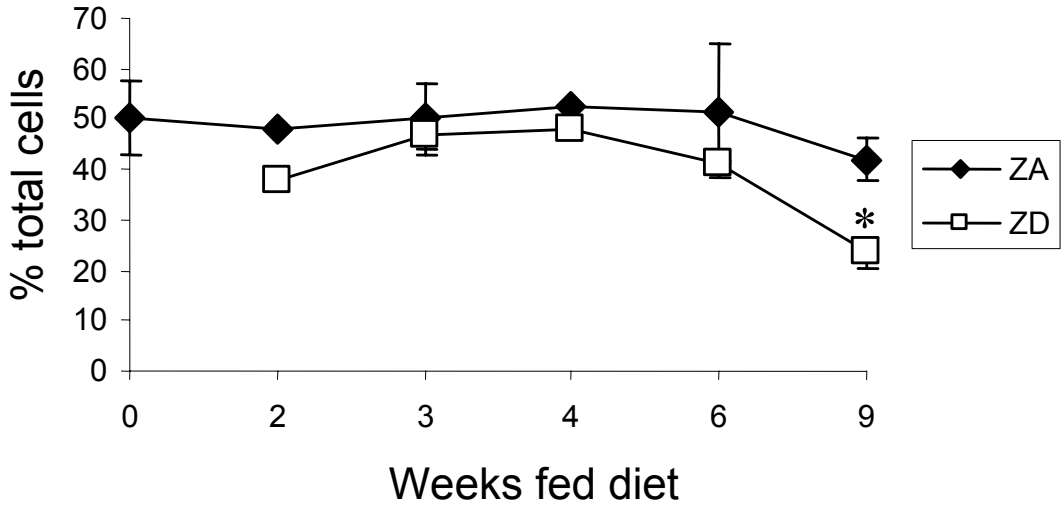


Figure 8. Mesenteric lymph node (MLN) CD3$^+$ CD28$^+$ lymphocytes. MLN CD3$^+$ CD28$^+$ lymphocytes as a percentage of the total recovered cell population in BALB/c mice fed a zinc-adequate (ZA) or zinc-deficient (ZD) diet. **P*<0.05, ZD vs. ZA at week 9. Data represent the mean ± SEM, n=1 to 6 mice per data point.

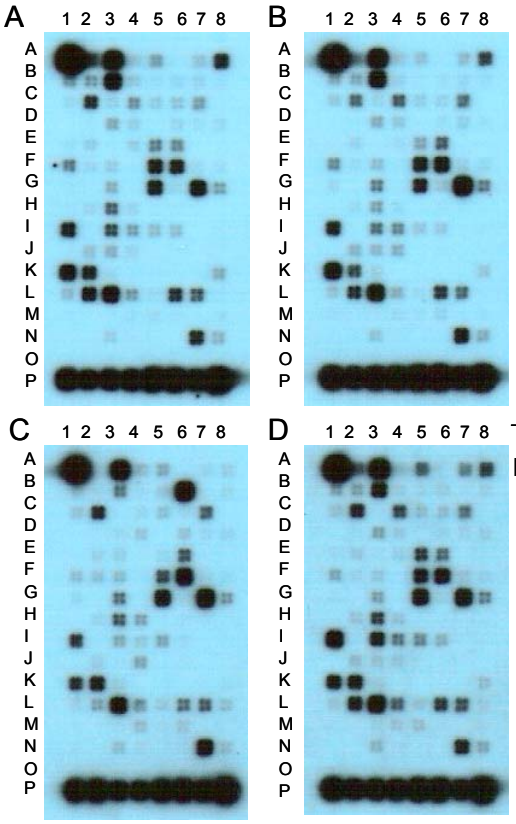
Microarray Analysis

Agarose gel electrophoresis of mouse colonic RNA samples isolated for use in microarray showed that RNA prepared by this method electrophoresed to show two ribosomal RNA bands, 28S and 18S. Bands in all lanes were sharp and the 28S band was of higher intensity than the 18S band, suggesting that the

RNA preparation was intact, had not been degraded, and was of high quality. Genes most changed were determined by visual examination of microarrays, evaluation of raw numbers following normalization with selected housekeeping genes (GEASuite), and fold changes determined by GEASuite scatterplot analysis. Figure 9, A and B, show the microarrays for the week 3 ZA and ZD mouse samples. A differential pattern of gene expression was found between a ZA and ZD mouse at week 3 [Fig. 9A (ZA) and 9B (ZD)], indicating that changes in gene expression occur after only 3 weeks of diet consumption. Genes listed are those the most highly expressed in comparison to the alternative diet at the same time point. At week 3 more genes were overexpressed in the ZA mouse in comparison to a ZD mouse. Microarray results of the week 9 colonic samples are shown in Figure 9C (ZA) and 9D (ZD). At week 9 genes that were overexpressed in the ZD mouse outnumbered those genes overexpressed in the ZA mouse. The percentage of genes that changed more than 1.5 fold according to the GEASuite scatterplot analysis for the week 3 microarray comparisons of a ZA versus ZD mouse colonic sample was 36%, in contrast to a total of 52% of genes differentially expressed for the same week 9 microarray comparisons.

mRNA Expression of Chemokine Ligands and Interleukins

Specificity and integrity of the designed primers was confirmed by the observation of a single product from the melt curves. Relative levels of colonic mRNA transcript levels were obtained by normalization of mRNA transcript levels for the gene of interest with 18S rRNA transcript levels run simultaneously. Relative CCL25 mRNA levels were lower in ZD mice at week 9 versus ZA mice (Fig. 10, P=0.057). Relative mRNA transcript levels for CCL17, CCL8, IL-18,



	Position	Genes Differentially Expressed
Panel E: Wk 3	K2	Interleukin 6 signal transducer (IL-6st)
	H3	Interleukin 13 receptor, alpha 1 (IL-13 Ra1)
	C2	Chemokine ligand 6 (CCL6)
	A2	Burkitt lymphoma receptor 1 (Blr1)
	I3	Interleukin 1 beta (IL-1β)
	C7	Chemokine receptor 2 (CCR2)
	C4	Chemokine ligand 8 (CCL8)
	E6	Chemokine ligand 14 (CXCL14)
	G7	Interleukin 12A (IL-12A)
Panel F: Wk 9	B6	Chemokine ligand 25 (CCL25)
	H4	Interleukin 15 (IL-15)
	G3	Interleukin 10 (IL-10)
	A8	Chemokine ligand 19 (CCL19)
	L2	Lymphotoxin B (LT-b)
	I3	Interleukin 1 beta (IL-1β)
	C4	Chemokine ligand 8 (CCL8)
	E5	Chemokine ligand 13 (CXCL13)
	B3	Chemokine ligand 21a (CCL21a, Serine)
	I1	Interleukin 18 (IL-18)

Figure 9. Colonic gene expression. Colonic gene expression was evaluated using a pathway specific Oligo GEArray Mouse Inflammatory Cytokines & Receptors Microarray (Superarray Bioscience) at 3 weeks (9A and 9B) and 9 weeks (9C and 9D) of diet in zinc adequate (ZA, 9A and 9C) and zinc deficient (ZD, 9B and 9D) BALB/c mice. Each microarray represents the RNA isolated from a single mouse. Genes listed in the tables to the right are those most differentially expressed in ZD vs. ZA mice at week 3 (9E) and week 9 (9F) of diet consumption after normalization with selected housekeeping genes.

and IL-1β were not different between ZA and ZD mice at week 9 in colonic tissue (Fig. 10).

Relationships between cytokines, chemokines, serum zinc, and metallothionein concentrations were evaluated through the use of correlations. CCL25 and CCL17 positively correlated with MT mRNA levels in ZA and ZD groups combined at 9 weeks [correlation coefficient (r)=0.85, *P*=0.02, n=7 and r=0.80, *P*=0.02, n=8, for CCL25 and CCL17, respectively], but neither correlated

with serum zinc. Although relative mRNA levels of IL-18 were not higher in ZD than ZA colonic tissue at week 9, relative IL-18 mRNA transcript levels positively correlated with the $CD3^+$ $TCR\gamma\delta^-$ $CD8\beta^+$ lymphocyte subpopulation as a percentage of total cells in the ZA and ZD groups (r=0.81, *P*=0.049, n=6). Furthermore, in the ZD mice IL-18 mRNA transcript levels positively with MT mRNA levels (r=0.96, *P*=0.04, n=4).

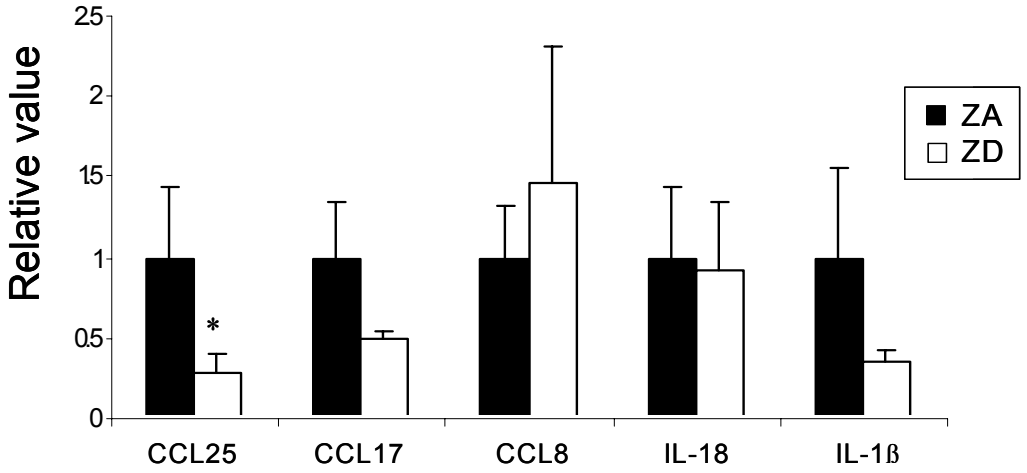


Figure 10. Colonic chemokine/cytokine/receptor mRNA. Relative transcript levels in colon of BALB/c mice 9 weeks after a zinc-adequate (ZA) or zinc-deficient (ZD) diet. mRNA expression was evaluated for chemokine ligand (CCL) 25, CCL17, CCL8, interleukin (IL) 18, and IL-1β with q RT-PCR and normalized to 18s rRNA. Data represent mean ± SEM, n=4/diet group. *P=0.057, ZD vs. ZA group.

DISCUSSION

Although previous studies showed alterations of cell populations and cytokines during zinc deficiency (13,16), the assessment of these changes occurred outside the GALT. In addition, numerous studies have demonstrated the symptomatic effects of zinc deficiency, such as parasitic infections, diarrhea, and intestinal morphological changes (14,105,106,131). These symptoms of zinc deficiency are specifically disruptive to the gastrointestinal tract, yet no studies have evaluated the cellular phenotypic changes, or cytokine and chemokine levels in the colonic tissue. The purpose of this study was to determine whether a progressive zinc deficiency altered T-lymphocyte subpopulation distribution or cytokines and chemokines within the GALT. This study demonstrated that a progressive zinc deficiency changed T-lymphocyte subpopulations distribution within the GALT and altered expression of cytokine genes in mouse colonic tissue, the site where many symptomatic effects of zinc deficiency are primarily localized.

Data from this study showed that BALB/c mice fed a ZD diet less than 1 ppm for 9 weeks had no decrease in thymic weight or body weight in comparison to mice fed a ZA diet. These data disagree with previously discussed work in A/J mice but are supported by a few studies in BALB/c mice (132,133). Interpretation of these studies is complicated because most of these incorporate an immune challenge into their protocol. Two studies, Minkus et al. (133) and Boulay et al.

(132), found that zinc diets containing 3-5 ppm zinc supported normal growth, Boulay et al. showed no effect of a ZD diet on thymus weight, and neither study showed that ZD mice had impaired immune responses to a nematode challenge. In contrast to these studies, several studies by Shi et al. (73,102) in BALB/c mice showed that a ZD diet containing less than 1 ppm zinc, resulted in impairment of weight gain in BALB/c mice and a subsequent inability to mount an effective immune response to nematode challenge. Strand et al. (134) found that addition of a stress, a pulmonary pneumococcal challenge, to the BALB/c model resulted in weight loss in a low zinc diet (less than 2 ppm). Data in BALB/c mice are inconclusive, and may depend on the particular stress and level of zinc deficiency to which the mice are exposed. These data suggest that zinc deficiency in some BALB/c models had no effect on body or thymus weight, but since most of these models contain an immune challenge component the timing and type of challenge may make the interpretation of the data difficult.

Additional factors contributing to the lack of effect of zinc deficiency on body and thymus weights in the present study could be genetic differences between strains and the housing conditions instituted at the University of Florida. The studies in which zinc deficiency consistently leads to decreased thymic weight and body weight losses in the absence of a stress were done in female A/J mice. A/J mice have T-cell responses distinguished by a high level of T_H1 cytokines (135). Prasad et al. (13) suggest that zinc deficiency has a bigger impact on the T_H1 cytokine profile, suggesting that a T_H1-biased strain may be more impacted by zinc deficiency. BALB/c mice, which appear to have less defined weight loss

effects during zinc deficiency, are a T_H2-biased strain (136). BALB/c mice may therefore be less susceptible to zinc deficiency. It was also shown that there are genetic differences in retention of ^{65}Zn when exposed to a ZD diet (137). Outbred mice retained more and excreted less ^{65}Zn following exposure to a ZD diet than a T_H1-strain (137). One final consideration is the newer husbandry practices in rodent care at universities. Many initial studies were done prior to the SPF conditions under which most mice are now shipped and maintained through acclimation. In this study, once the mice were given the experimental diet they were housed individually in stainless steel wire hanging cages; however, individuals entering the area still followed SPF gowning procedures. The mice are therefore exposed to fewer immune challenges in more recent studies than older studies. This may explain the differences obtained in body and thymus weights for the A/J mice in the studies described above. If housing procedures vary at different institutions this may explain some of the inconsistencies in data obtained with BALB/c mice. In evaluation of weight differences between ZA and ZD mice in different studies it is important to consider the zinc concentration of the diet and weeks mice were fed the diet, strain of mice, and housing conditions (indicating potential pathogen exposure).

Liver weight was found to be unaffected by zinc status in the current study. Previous studies evaluating the effect of zinc status on liver weight are contradictory. Again, this may be because many zinc deficient studies incorporate an immunological challenge making the data difficult to interpret. Zhou et al. (138) found that rats fed a marginal ZD diet of 7 ppm for 5 weeks

showed no change in liver weight. In contrast, Shi et al. (101) found that female BALB/c mice fed a 0.75 ppm ZD diet had decreased liver weight at all timepoints weeks 6-9. Interestingly, in these mice the ZD diet also led to weight loss, loss of hair, decreases in thymus weight, along with the decreased liver weight at week 7-9 (101). Furthermore, a subset of these mice exposed to a nematode challenge showed the ZD mice had increased worm burdens and were thus more susceptible to parasitic challenge (101). Boulay et al. (132) showed that during a primary infection against a nematode, BALB/c mice fed a 3 ppm ZD diet had increased liver weight compared to mice fed the 60 ppm ZA diet.

The zinc deficient status of the mice was confirmed by serum zinc levels that were 64% lower in ZD mice than the ZA mice. To further access the zinc status of the mice used in this study, MT mRNA levels were analyzed. mRNA levels for MT, a zinc-regulated zinc storage protein, decreased during zinc deficiency in agreement with previous studies (71,72,118). These data confirm that the zinc deficient mice were indeed zinc deficient.

Data from this study showed that there was no affect of 9 weeks of diet on the total number of cells extracted from each of the tissues evaluated, i.e. mesenteric lymph nodes, Peyer's patches, IEL, LPL, and cIEL. Extracted IEL and cIEL were passed through nylon wool columns, which are intended to remove contaminating B cell populations and epithelial cells; therefore, contrary to the other tissues evaluated (mesenteric lymph nodes, Peyer's patches, and lamina propria lymphocytes) whose numbers reflect the entire lymphocyte populations and some additional leukocytes, IEL and cIEL numbers should be representative

of a more purified T-lymphocyte population. No studies to date have evaluated the effect of zinc deficiency on GALT numbers; however, systemically zinc deficiency is often associated with decreased lymphocyte populations. King et al. (15) showed that this systemic lymphopenia in zinc-deficient A/J or CAF1/J mice is associated with a 34-38% thymic involution and a corresponding 50-300% increase in apoptosis of pre-T cells. Similar apoptotic mechanisms are thought to have the same affect on pre-B cells in the marrow, leading to a loss of pre-B cells in zinc deficiency (139). The lack of changes in the total number of cells extracted from the tissues in this study is consistent with the lack of change in thymus weight observed in these data. The failure to observe changes in cellular numbers is in agreement with King and Fraker who showed no overall cell number changes, only a redistribution of cellular populations (15,70). The inability to see observable differences in total cell numbers in the GALT extracted from ZA and ZD mice suggests that even if systemic changes did occur, these changes were not reflected in the tissue cellular concentrations. Additionally, zinc deficiency in the strain of mice used in the current study may not have induced systemic changes in cell numbers in contrast to data obtained in other strains of mice.

Flow cytometric analysis indicated a larger percentage of $CD3^+$ cells were found in IEL isolated from the colonic tissue of zinc-deficient mice. The higher percentage of $CD3^+$ $TCR\gamma\delta^-$ $CD8\beta^+$ cells isolated from cIEL of ZD mice may account for the observed increase in the $CD3^+$ population. The corresponding decrease in this $CD3^+$ $TCR\gamma\delta^-$ $CD8\beta^+$ cell population in the Peyer's patches and

other CD8β$^+$ populations in the Peyer's patches and MLN in ZD mice may indicate a movement of this cell population from Peyer's patches and MLNs of the gastrointestinal tissue into the cIEL. The TCRγδ$^-$ CD8β$^+$ cell population, which was elevated in the cIEL and decreased in the Peyer's patches most likely represents conventional cytotoxic TCRαβ$^+$ CD8αβ$^+$ lymphocytes, which are typically associated with peripheral immune function and the development of intestinal inflammation. These data are in agreement with Hosea et al. (17) who found a higher percentage of TCRαβ$^+$ CD4$^-$ CD8$^+$ in thymocytes from ZD rats. A decrease in TCRγδ$^+$ lymphocytes and increase in CD8αβ$^+$ lymphocytes are associated with increased proliferative capacity and IFN-γ production, both proinflammatory conditions (140). TCRαβ$^+$ CD8αβ$^+$ populations in the IEL often also have CD8αα, a marker typically associated with IEL or cIEL. In this study we did not determine the CD8αα status of the TCRαβ$^+$ CD8αβ$^+$ population. CD8αβ and CD8αα have different sensitivities to antigens, attributed to the chains they express on their surface and the signaling cascades that are activated following cell stimulation (141). The β chain increases the sensitivity of the TCR, potentially contributing to the cytotoxic activity of CD8αβ$^+$ cells (142). IELs expressing CD8αα have higher levels of Bcl-2 (a protein that is protective against apoptosis), lower expression of costimulatory markers (like CD28), and lower production of pro-inflammatory cytokines (141,143,144).

The only changes in the TCRγδ$^+$ lymphocyte population observed in this study were in the Peyer's patches of ZD mice where this population increased. This cell population may represent the TCRγδ$^+$ CD8αα$^+$ lymphocytes that are

thought to play a role in epithelial maintenance. Komano et al. (145) showed that TCRγδ$^{-/-}$ mice failed to regenerate intestinal epithelial cells that are constantly turning over in the gut mucosal epithelium and therefore determined that TCRγδ^{+} lymphocytes are essential for maintaining mucosal integrity. This was further supported by the work of Chen et al. (146) who showed that the production of keratinocyte growth factor by TCRγδ^{+} cells prevented dextran sodium sulfate induced colitis.

CD4^{+} lymphocytes were lower in cIEL of ZD mice, and the double positive CD8β^{+} CD4^{+} (most likely an immature pre-T-cell population) was lower in the Peyer's patches of ZD mice. These findings are in agreement with the systemic measurements of King et al. (15) which showed an increase in apoptosis of the CD8β^{+} CD4^{+} cells and subsequent decreases in this pre-T-cell population in ZD mice. The data in the current study suggest that some of the systemic changes occurring within zinc deficiency are perpetuated into the GALT. Overall, these data suggest that within the GALT, zinc deficiency has the greatest impact on conventional T-cell populations, such as TCRγδ^{-} CD8β^{+} and CD4^{+} lymphocytes.

Changes were observed in this study in the CD28 T-cell costimulatory receptor. The current data show Peyer's patches from ZD mice had reduced CD3^{+} CD28^{+} CD4^{-} CD8β^{+} populations as a percentage of total T cells (CD3^{+} cells), this may account for the decreased CD8β^{+} population in this tissue from the ZD mice discussed above. These data also showed the CD3^{+} CD4^{+} CD28^{+} subpopulation decreased in cIEL isolated from ZD versus ZA mice, this population change may be responsible for the decrease identified in this study in

the $CD3^+$ $CD4^+$ cIEL population. These data indicate that the Peyer's patch $CD3^+$ $CD8\beta^+$ and cIEL $CD3^+$ $CD4^+$ subpopulation losses were specifically also $CD28^+$.

The tissue which expressed the largest number of T-cell costimulatory marker changes was the mesenteric lymph nodes. Although no overall $CD3^+$ changes were identified in the MLN T-cell population independent of other markers, decreases were identified in the $CD3^+$ $CD28^+$ cell population from ZD mice. These data suggest that although zinc deficiency does not decrease the overall T-cell population, the T cells that are present express less $CD28^+$ costimulatory receptor and are therefore less activateable suggesting a possibility of lack of stimulation potential or anergy. The $TCR\gamma\delta^+$ $CD8\beta^-$ $CD28^+$ $CD3^+$ MLN subpopulation was lower in the ZD than the ZA mice, although the $TCR\gamma\delta^+$ $CD8\beta^-$ $CD3^+$ subpopulation remained unchanged during ZD. This decrease identified in a $CD28^+$ subpopulation of the $TCR\gamma\delta^+$ T-cell population may not represent a large population. The $TCR\gamma\delta^+$ population composed 7.0 ± 0.48% of the $CD3^+$ cell isolated from the mesenteric lymph node in this study and according to Helgeland et al. (38) accounts for only 1% of total cells isolated from MLN of rats whose MLN is mainly composed of the conventional T-cell populations. Of all of the tissues evaluated only one $CD28^+$ population, the $TCR\gamma\delta^-$ $CD8\beta^+$ $CD3^+$ population, in the MLN was found to increase; however, it may not constitute a large population since decreases were obtained for the overall $CD8\beta^+$ $CD3^+$ MLN population (discussed above) independent of the other cell markers. These data suggest that decreases in the $CD28^+$ subpopulation of the conventional T-cell populations, $CD4^+$ (cIEL), $CD8\beta^+$ (Peyer's patch), and

$CD3^+$ (MLN), were found to be the most affected by zinc deficiency and may be contributing to the losses in these T-cell subpopulations. This study did not identify changes in the CD28 status of the overall $CD3^+$ population in the cIEL or in $CD3^+$ TCRγδ$^-$ CD8β$^+$ cells, both of which were shown in the current study to increase in the cIEL during zinc deficiency.

Previous studies found a lack of CD28 expression in aged patients or individuals with chronic inflammatory diseases (147,148). The $CD28^-$ T-cell population increases with increasing age, with newborns expressing only about 1% of $CD28^-$ T cells, while centenarians express approximately 30% $CD28^-$ T cells (148). Patients with rheumatoid arthritis had higher percentages of circulating $CD4^+$ $CD28^-$ and $CD8^+$ $CD28^-$ than controls (149). The level of expression of CD28 has also been shown to decrease in Chagas' disease (a parasitic infection that can cause cardiac dysfunction) and Crohn's disease (150,151). Stimulation of T cells in the absence of CD28 results in non-responsiveness in the T cell and Effros et al. (152) suggested that this may lead to a state of anergy. The progressive lack of CD28 expression due to zinc deficient status in the current study may be indicative of an inflammatory tendency without progression to an observable disease state, and may provide insight into a potential mechanism for mucosal barrier dysfunction as a result of zinc deficiency.

CD28 is an essential disulfide-linked homodimer on the surface of T cells following interaction of the TCR with the antigen/MHC or MHC-like complex. The interaction of CD28 with its ligand is essential for the proliferation of the T cell,

production of IL-2, and formation of cytotoxic T cells (152). Azuma et al. (148) found that in the circulating TCRαβ T-cell population of healthy adults $CD4^+$ $CD28^-$ T cells were uncommon; however, $CD8^+$ $CD28^+$ and $CD8^+$ $CD28^-$ were present at a ratio of approximately 2:1. There are few systemically circulating TCRγδ T cells, but the authors did identify the presence of both $CD28^+$ and $CD28^-$ varieties within this T-cell subset (148). Evaluation of $CD3^+$ $CD28^-$ T-cell population locations (they were absent in the thymus and decreased in cord blood), large size, and additional markers (CD54, CD58, and CD11a), led the authors to suggest that these cells may represent a memory T-cell population; however, further evaluation of CD45RO expression, the memory T-cell marker, did not identify differences for the memory marker between the $CD3^+$ $CD28^+$ and $CD3^+$ $CD28^-$ populations (148). Garcia de Tena et al. (150) did identify increases in the CD45RO marker in $CD4^+$ $CD28^{null}$ T cells in patients with active Crohn's disease, indicating that this population may represent a memory T-cell subpopulation. Functional inconsistencies also make determination of the $CD28^-$ T-cell activity difficult. $CD3^+$ $CD28^-$ cells may be considered anergic because they showed lower proliferation, less than 5% expressed cytoplasmic IL-2 in response to anti-CD3 antibodies, and they had less markers of activation (such as CD69 and CD25) (148). This was in contrast to the $CD3^+$ $CD28^+$ cell population which had higher proliferative responses, cytoplasmic IL-2 present in 65% of their population, and expressed activation markers (148). Frydecka et al. (153) found that $CD28^+$ populations of $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ were lower in patients with B-cell chronic lymphocytic leukemia than healthy control subjects. In

addition, this cell population was slower to activate (as measured by CD28 density on the cell) and proliferate in response to stimulation in culture in comparison to the same populations isolated from healthy control subjects, indicating a hyporesponsiveness (153). These data are inconsistent with the finding that $CD3^+$ $CD28^-$ cells have greater cytotoxic activity than their $CD28^+$ counterparts (148). $CD4^+$ $CD28^-$ T cells have been shown to be highly autoreactive, and Vallejo et al. (147) suggest that altering the CD28 subset can lead to “disease related immune abnormalities”. Although the mechanism by which the $CD28^-$ T-cell population causes immune dsyregulation is unknown, their increased presence in a number of diseases is well documented. The loss of $CD28^+$ T-cell subpopulations in the Peyer’s patches, mesenteric lymph nodes, and cIEL of ZD mice in the current study provides a potential mechanism for the disruption of the mucosal barrier that occurs in zinc deficiency. These data suggest that zinc deficiency may be associated with impaired T-cell activation within the GALT.

Several mechanisms have been proposed for the modulation of CD28 expression in the above referenced studies. One theory is that the loss of CD28 is due to replicative senescence, or cells that have reached their Hayflick Limit, referring to the maximum number of cell divisions possible for a particular cell (152,154). This is supported by cell culture analysis which showed decreases in $CD28^+$ cells with increased population doubling (155). Effros et al. (152) proposed that this replicative senescence is due to telomere shortening (loss of DNA) during progresssive rounds of cell division. This theory is supported by

Monteiro et al. (156) who found that telomeres from $CD8^+$ $CD28^-$ T cells were 1.4 kb shorter than those from $CD8^+$ $CD28^+$ T cells, and estimated that the $CD28^-$ T cells had experienced 14 to 28 more population doublings than the $CD28^+$ T cells. Vallejo et al. (147) proposed that in chronic disease states this replicative senescence eventually leads to immune dysfunction. In addition to telomeric shortening, expression of CD28 is downregulated by TNF-α as previously described (127,153). Bryl et al. (127) using annexin and CSFE staining showed that TNF-α triggered decreases in CD28 was not due to apoptosis or replicative senescence, respectively. The authors showed that TNF-α downregulated CD28 expression by decreased transcription through inhibition of the binding of nuclear proteins to the CD28 gene promoter region (127). Furthermore, it was mentioned above that patients with rheumatoid arthritis had decreased $CD28^+$ expression on their $CD4^+$ and $CD8^+$ cells (149). Bryl et al. (149) showed that administration of anti-TNF-α treatment for 1 week resulted in a 69% increase in CD28 molecules, and 56% of subjects showed improvement in disease. In the case of a repeated antigenic exposure, as might occur due to decreased tolerance or low dose repeated antigenic stimulation, expansion of the $CD28^-$ population occurs as discussed in the Arosa et al. (50) studies above where the intraepithelial $CD8^+$ $CD28^-$ population expanded following incubation with epithelial cells functioning as antigen-presenting cells. One additional mechanism for the decreased CD28 subpopulations includes the loss due to apoptosis (157). However, this loss is not specific for cell surface markers such as CD28, but would include decreases in

CD3, CD4, CD8, and CD56 as well as other cell surface molecules as the whole cell is lost (157).

Insight into these mechanisms responsible for loss of CD28 expression and current knowledge about zinc deficiency allows speculation into the mechanism by which zinc deficiency may have caused CD28 losses in the current study. It was mentioned earlier that increased TNF-α is observed in zinc deficiency (98). Taylor et al. (158) showed that TTP-deficient mice (TTP is highly expressed in the intestine) developed dermatitis and chronic skin inflammation along with other phenotypic changes, and that administration of anti-TNF-α antibody reversed the TTP-deficient phenotype. This reduction in TTP and resulting increase in TNF-α that occurs in zinc deficiency may be responsible for the decreased CD28 in this work. Interestingly, zinc deficiency is associated with parakeratosis and dermatitis and hyperkeratosis with areas of parakeratosis were found in TTP-deficient and STAT/CD28 knockout mice, respectively (158,159). Additionally, since zinc deficiency has been shown to increase $p56^{lck}$ expression (72,117), this signal may be seen as a repeated stimulation or a primary signal with no subsequent secondary signal, leading to loss of other costimulatory molecules and resulting in the development of an anergic, $CD28^{-}$, cell population. The documented apoptosis that occurs in zinc deficiency may be an additional mechanism for CD28 loss, however, the CD3, CD4, CD8 populations in this study were not decreased in all tissues which had observed CD28 decreases as you would expect if these changes were due to apoptosis (157).

By using the pathway-centric microarray, a number of inflammatory-related genes whose expression in the colon appeared to be altered by 3 and 9 weeks of zinc deficiency were identified. Chemokines, cytokines and interleukins are proteins that act as mediators to orchestrate the homing and inflammatory responses of leukocytes. Their corresponding receptors are necessary for the interpretation of these signals by target cells. Relative expression levels of some of these genes were further assessed using q RT-PCR with 4 mice/group. q RT-PCR confirmed initial microarray data of decreased expression of CCL25 mRNA in ZD mice in this study. CCL25 is a chemokine ligand expressed on intestinal epithelial cells and the endothelial cells of the small venuoles in the intestinal lamina propria in mice (160). CCL25 interacts with the receptor CCR9, present on almost all T cells in the small intestine and on a fraction of IgA producing antibody-secreting plasma cells (160). Decreases between diet groups in this chemokine, important in homing, did not result in a decrease in overall numbers of IEL and LPL observed in this study (Fig. 4C, D, and E). One possible explanation is that differences in mRNA levels of CCL25 were observed but correlating changes in protein levels of CCL25 may not have been occurring. Future studies should evaluate CCL25 protein levels in colonic tissue during progressive zinc deficiency. The presence of IEL and LPL lymphocytes and IgA secreting plasma cells would be important in the response to inflammation or maintenance of the gut barrier. These data suggest a potential inability of ZD mice to produce sufficient transcripts for this essential chemokine ligand, leading

to possible reductions in IgA secreting plasma cells, alterations in the development of T and B cells, and a reduction in resident IEL T cells (65).

In addition to CCL25, other genes were selected for comparison of expression using q RT-PCR based on identification of differences on the microarray and for a variety of other reasons as well. For example, CCL17 (position A7, Fig. 9) was selected because the CCL17 gene maps to the same locus as MT (161), and therefore it is reasonable to hypothesize that it may be controlled by zinc as is MT. CCL17 mRNA was shown to be more highly expressed in the inflamed mucosa of patients with Crohn's disease (162). CCL-8 (Position C4, Fig. 9) was chosen because it is secreted by many cell types including epithelial cells, and has an important role in influencing the migration of T cells and monocytes during inflammation (163). Van Coillie et al. (164) showed that IFN-γ, produced by activated $CD8\beta^+$ cells (the population of cells which increased in the cIEL in ZD mice) and NK cells, cause upregulation of mRNA transcripts for CCL8. IL-1β (Position I3, Fig. 9) was selected because it provides an important cosignal in the activation of T lymphocytes, has a role in the induction of the T_H1 response, has anti-tumor affects, and also increases the subpopulation of $TCR\alpha\beta^+$ $CD8^+$ cells when used for in vivo proliferation (165). IL-18 (Position I1, Fig. 9) was chosen because it is a product of activated macrophages, increases the activity of NK and cytotoxic T lymphocytes (CTL), and like IL-1β has a role in the induction of the T_H1 response and anti-tumor properties (166,167). The effects of IL-18 are most potent when there is also costimulation with IL-12, this stimulates the activation of $CD8^+$ T cells and the

subsequent production of IFN-γ (168). Significant differences in CCL17, CCL8, IL-1β, and IL-18 gene expression were not obtained by addition of more animals per group for q RT-PCR analysis. A larger sample population is needed to assess the significance of any of these trends, for example, based on these data the number of mice required to yield a significant difference for the CCL17 based on 80% power and $P<0.05$ is 8 mice per group. The initial microarray data suggest the possibility that some of the cytokines, chemokines, and interleukins changed during the zinc deficiency, and this may play a role in the observed lymphocyte changes. It is also worth noting that the alternative can occur as well: changes in subpopulation distributions and activation can alter the cytokine, chemokine, or interleukin microenvironment.

Correlations were identified between several of the factors evaluated in this study. A positive correlation was found between CCL25 mRNA levels and MT mRNA levels, this correlation supports the finding that CCL25 mRNA levels were lower in ZD mice. MT was used in this study as a zinc status marker, because this zinc regulated gene is known to decrease in zinc deficiency; therefore, it is logical that levels of MT mRNA would positively correlate with CCL25 mRNA levels if CCL25 mRNA expression is dependent of zinc status. CCL17 mRNA levels did not appear to be affected by dietary zinc status. However, CCL17 mRNA levels did correlate with MT mRNA levels independent of dietary zinc status. This may be attributed to the fact that both genes are found in close proximity to each other (161). Data in this study showed that IL-18 mRNA levels positively correlated with the $CD3^+$ $TCR\gamma\delta^-$ $CD8\beta^+$ cell population. These data

are in agreement with several studies which have shown that increased IL-18 administration is associated with an increased $CD8^+$ T-cell population (169,170). Administration of an antigen along with an adjuvant in BALB/c mice downregulated airway inflammation and led to increased mRNA levels of IL-18 (169). However, administration of anti-CD8 antibody eliminated the beneficial reduction in the model of reactive airway disease, leading the authors to conclude that $CD8^+$ T cells played a role in this reduction (169). An association was made between the increased $CD8^+$ T-cell population and the increased IL-18 mRNA levels (169). Ju et al. (170) showed that genetically modified dendritic cells that overexpressed IL-18 when injected simultaneously with a tumor vaccine in C57BL/6 mice produced a higher percentage of tumor specific $CD8^+$ T cells than when the tumor vaccine was given alone. An IL-18 treated mixed lymphocyte culture stimulated with IL-2 produced a two-fold increase in CD8/CD4 ratio in comparison to an IL-2 stimulated non IL-18 treated culture, with the actual number of $CD8^+$ T cells increasing threefold (171). Furthermore, the IL-18 treated $CD8^+$ T cells upon stimulation had increased IFN-γ production, upregulated IL-2R alpha chain expression, and increased cytotoxic activity (171). Depletion of the $CD4^+$ T-cell population inhibited expansion of $CD8^+$ cells, indicating that IL-18 induced development of $CD8^+$ T cells is $CD4^+$ T-helper cell dependent (171). The correlations identified in the present study are consistent with expected results based on the literature and offer further validity to the data obtained.

Combining the information already known about changes that occur in zinc deficiency with the data presented in this study, a mechanism can be proposed

for the redistribution of the cellular populations including changes in the cIEL populations (increased overall $CD3^+$ and $CD8\beta^+$, and decreased $CD4^+$), in Peyer's patches populations (decreased $CD8\beta^+$ and $CD4^+$), in $CD28^+$ populations, for the changes in cytokines, chemokines, and receptors found via microarray, and for the change in CCL25 confirmed on q RT-PCR (Fig. 11). In the major cell populations for which decreases were identified in this study, i.e. in CD28 populations, $CD3^+$ $CD28^+$ $CD4^-$ $CD8\beta^+$ (Peyer's Patches), $CD3^+$ $CD4^+$ $CD28^+$ (cIEL), and $CD3^+$ $CD28^+$ (MLN, CD4 or CD8 status undetermined), zinc deficiency could lead to dysfunctional TTP (a zinc-finger protein which makes TNF-α mRNA unstable) and could lead to increased TNF-α, as previously shown to occur in zinc deficiency (98,124,126). TNF-α then has the potential to disrupt the transcription of CD28 via alteration of protein binding at the promoter (127). At the same time the $p56^{lck}$ expression, associated with the CD4 or CD8 molecule, is increased in zinc deficiency (72,117); however, the absence of CD28 expression on these cells possibly prevents the formation of a lipid raft (172). Inability to form the lipid raft results in failure of the $p56^{lck}$ kinase to phosphorylate ZAP-70, resulting in no onward perpetuation of the signaling cascade, and a decrease in the transcription within the nucleus. The receipt of the primary signal increased $p56^{lck}$, and absence of receipt of a secondary signal may render these cells anergic. Alternatively, the decreased availability of zinc may prevent the $p56^{lck}$/CD4 or CD8 metal binding complex to form and prevention of secondary signal transduction (119). Furthermore, van den Brandt et al. (173) showed that CD4 or CD8 cells lacking the costimulatory molecule

CD28 were more sensitive to glucocorticoid induced apoptosis. Since glucocortocoids are known to increase in zinc deficiency (76), it is in this manner that the cell population identified above may decrease. The lack of a function in these cell populations, as a result of anergy or apoptosis, may alter chemokine and cytokine microenvironment. Alterations in cytokines and chemokines can influence the distribution of cellular populations within the GALT.

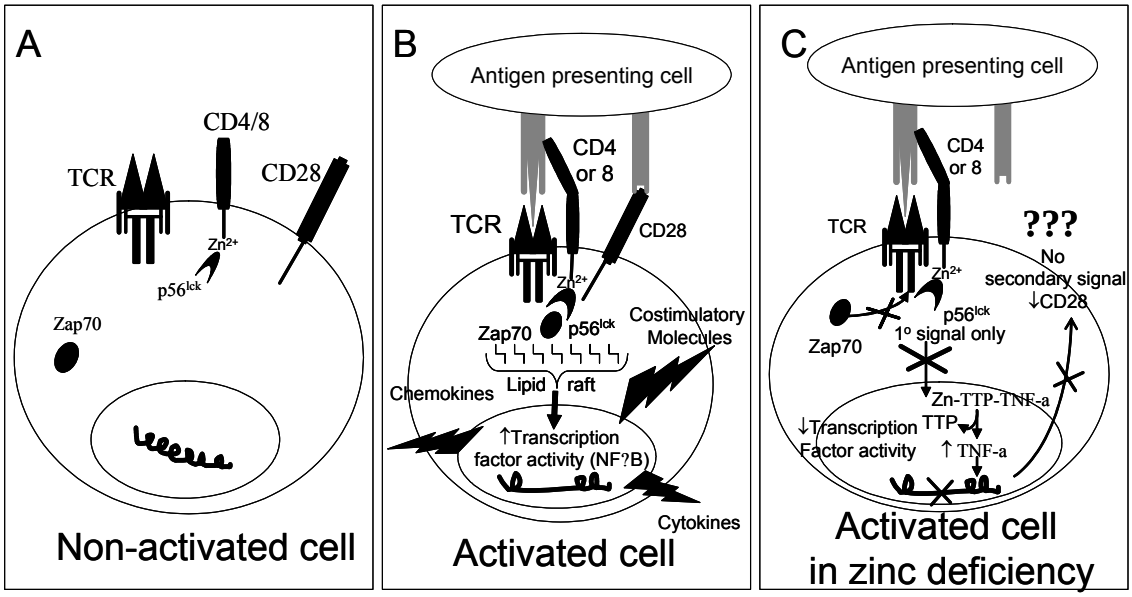


Figure 11. Mechanism for CD4 and/or CD8 cellular changes during zinc deficiency. Panel A shows the non-activated cell, with the T-cell receptor (TCR), CD4 or 8 molecule, and the CD28 costimulatory molecule. The Zap-70 is not associated with the cytoplasmic tail of the receptor in the non-activated cell, $p56^{lck}$ is not active, and the lipid raft is not formed. The activated cell (Panel B) has engagement of the TCR and CD28, allowing proper signaling and formation of the lipid raft. The $p56^{lck}$ that is assocated with the CD4 or CD8, activates Zap-70 which continues this signaling cascade and results in increased transcription. In zinc deficiency (Panel C) the cell TTP becomes destabilized, increasing TNF-α, decreasing the transcription of CD28 (98,124,125). This reduction in the costimulatory molecule CD28, along with increased $p56^{lck}$ results in a primary but no secondary signal, failure to form the lipid raft, and lack of ZAP-70 transmission of signal (72,117,172). This lack of CD28 may result in anergy or increased sensitivity to glucocorticoid-induced apoptosis, altering the cytokine/chemokine microenvironment (173).

Potential consequences of the changes that occur in zinc deficiency when the data in this study are considered in light of what is already known to occur in zinc deficiency causes concern. In this study increases within the colon in the proinflammatory population CD8β (due to potential infiltration from the Peyer's patches and MLNs), decreases in the conventional CD4 population and CD28 costimulatory molecule, and failure to produce transcripts for CCL25 suggest an increase in inflammatory potential occurring simultaneous to decreased ability to respond to immune challenges. This decreased presence of the conventional CD4 T-helper cell population, costimulatory molecule CD28 necessary for proper signaling, and decreased ability to influence homing via CCL25 mRNA expression may impair the ability of a zinc-deficient animal/human to respond to a mucosal challenge. In this way zinc deficiency tips the scale in priming the system towards potential inflammation, so that when a GALT challenge is encountered the mucosal immune system is more easily overcome (Fig. 12).

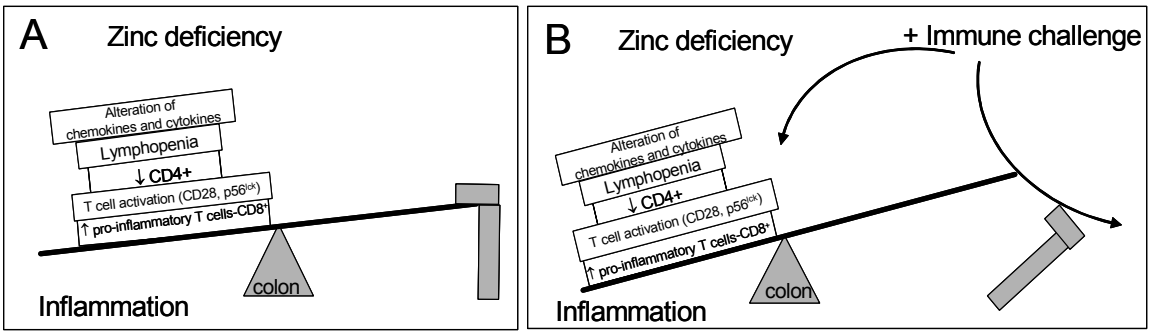


Figure 12.Proposed model. Preliminary data suggest that zinc deficiency in the absence of an immune challenge (12A) is associated with changes in T-lymphocyte subtypes, inflammatory chemokine and cytokine and/or their receptor/ligands, and T-cell activation markers that increase the susceptibility of the intestinal mucosa and associated lymphoid tissue to inflammation. Zinc deficiency with a subsequent immune challenge (12B) could tip the balance toward an overwhelming inflammatory response and tissue injury.

These data provide insight into the pathway through which zinc deficiency may increase susceptibility to parasitic challenge and become associated with increased gastrointestinal disorders.

Future studies in the area of zinc deficiency and mucosal immunity should follow-up on some of the differences identified in this work. This study identified changes in T-cell subpopulations within the GALT tissue unique from those occurring systemically, along with GALT changes in the cytokine and/or chemokine expression as a result of zinc deficiency. A mechanism for the potential cytokine and/or chemokine driven T-cell subpopulations changes should be evaluated for specific cellular populations important for protection from the effects of zinc deficiency. Furthermore, $p56^{lck}$ and TNF-α levels should be evaluated simultaneously with CD28 expression to identify the potential mechanism of CD28 decreased expression in ZD mice. An interesting observation in this study was that male BALB/c mice did not experience any weight loss as a result of the zinc deficiency. Due to the T_H2-biased responses of the BALB/c strain of mice, if the mechanism by which a zinc deficiency exerts its symptomatic effects is via T_H1 pro-inflammatory cytokines, BALB/c mice might be more resistant to the effects of a zinc deficiency and it might be beneficial to repeat this work in a T_H1-biased strain. Since many of the side effects of zinc deficiency occur in situations where the gut is exposed to pathogens, the addition of a gut stress, and differential responses by T_H1- and T_H2-biased mouse strains may provide insight into the pathways leading to breakdown of the gut barrier that occurs during zinc deficiency. The addition of antibodies against cytokines

and/or chemokines that may evoke cellular changes may provide future therapies to alleviate the negative consequences of zinc deficiency. The BALB/c strain used in this study could be beneficial in that it allows the researcher to create a chronic zinc-deficient mouse model for experimentation without induction of negative side effects such as diarrhea and parakeratosis. Further studies in this lab will confirm changes in gene expression as documented by the microarray, evaluate the resistance BALB/c mice to the outward symptoms of zinc deficiency in comparison to other strains, evaluate differences in the expression of cytokines, chemokines, and receptors between ZA and ZD mice with the addition of a stress to this model, and probe for the mechanism leading to CD28 T-cell receptor changes.

These data suggest that zinc deficiency alters cellular subpopulations and genes involved in the regulation of pathways important in protection of the mucosal gut barrier. These cellular changes may be the result of changes in the cytokine, chemokine, and/or their receptors in the microenvironment, which have the potential to selectively attract and stimulate different lymphocyte populations. The mice in this study were exposed to a zinc-deficient diet for 9 weeks, sufficient to cause decreases in serum zinc levels and relative colonic MT levels, alterations to the GALT cytokine and chemokine microenvironment, and changes to conventional T-cell populations along with their costimulatory marker CD28, but the ZD mice never experienced the other expected symptoms of severe zinc deficiency as described in previous studies with other strains of mice (139). A

long-term zinc deficiency with no immune challenge in male BALB/c mice may be more comparable to a moderate zinc deficiency in other strains of mice.

Summary and Conclusions

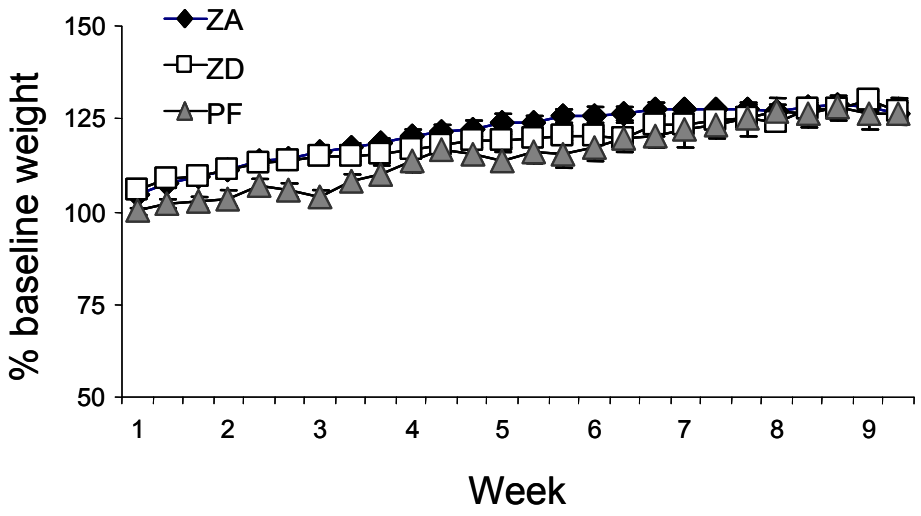
The altered microenvironment in the GALT during a 9-week progressive zinc deficiency results in changes in T-cell subpopulations within the MLN, small intestinal Peyer's patches, and cIEL. These results also indicate that zinc deficiency alters colonic expression of chemokines, cytokines, and their receptors important in inflammatory pathways. Furthermore, these results show a decreased presence of the T-cell costimulatory activation marker CD28. Zinc deficiency is associated worldwide with an increase in parasitic infections. During chronic inflammatory diseases, such as viral and parasitic infections, it has been shown that there is an increased percentage of $CD8^+$ $CD28^-$ T cells (50). Therefore, alterations in chemokines and cytokines, and in T-cell subpopulations along with their decreased activation potential, may lead to increased susceptibility to a mucosal barrier disruption and subsequent inflammation of the intestinal tissue. Previous evidence for the disturbance of the mucosal barrier in zinc deficiency includes the increased incidence of parasitic infections, morphological changes identified by histology, increased mRNA for TNF-α, and increased iNOS. Future research should focus on these altered T-cell subpopulations and identify the potential mechanism by which zinc deficiency affects these T-cell populations.

APPENDIX A
DIET COMPOSITION

	Zinc Adequate 0.85 ppm Zinc		**Zinc Deficient 30 ppm Zinc**	
%	gm	kcal	gm	kcal
Protein	20	20.5	20	20.5
Carbohydrate	66.3	68	66.3	68
Fat	5	11.5	5	11.5
Total		100		100
kcal/gm	3.9		3.9	
Ingredient	**gm**	**kcal**	**gm**	**kcal**
Egg Whites, Spray Dried	200	800	200	800
Corn Starch	150	600	150	600
Sucrose	502.6	2010.4	502.5	2010.2
Cellulose, BW200	50	0	50	0
Corn Oil	50	450	50	450
Mineral Mix S19401	35	0	35	0
Vitamin Mix V10001	10	40	10	40
Choline Bitartrate	2	0	2	0
Biotin, 1%	0.4	0	0.4	0
Zinc Carbonate, 52.1% Zinc	0	0	0.056	0
Total	**1000**	**3900**	**1000**	**3900**

Diet contents were obtained from Research Diets.

APPENDIX B
WEIGHT DATA OF ZINC ADEQUATE, ZINC DEFICIENT, AND PAIR-FED DIET GROUPS



APPENDIX C
MOUSE INFLAMMATORY CYTOKINE AND RECEPTORS MICROARRAY KEY

Microarray Position Map

Gapdh 1	Blr1 2	C3 3	Ccl1 4	Ccl11 5	Ccl12 6	Ccl17 7	Ccl19 8
Ccl2 9	Ccl20 10	Ccl21a 11	Ccl22 12	Ccl24 13	Ccl25 14	Ccl3 15	Ccl4 16
Ccl5 17	Ccl6 18	Ccl7 19	Ccl8 20	Ccl9 21	Ccr1 22	Ccr2 23	Ccr3 24
Ccr4 25	Ccr5 26	Ccr6 27	Ccr7 28	Ccr8 29	Ccr9 30	Cx3cl1 31	Cx3cr1 32
Cxcl1 33	Cxcl10 34	Cxcl11 35	Cxcl12 36	Cxcl13 37	Cxcl14 38	Cxcl15 39	Cxcl2 40
Cxcl4 41	Cxcl5 42	Cxcl9 43	Cxcr3 44	Fcer1g 45	Fcgr1 46	Gpr2 47	Ifna2 48
Ifng 49	Igh-4 50	Il10 51	Il10ra 52	Il10rb 53	Il11 54	Il12a 55	Il12b 56
Il12rb2 57	Il13 58	Il13ra1 59	Il15 60	Il16 61	Il17 62	Il17b 63	Il17e 64
Il18 65	Il1a 66	Il1b 67	Il1r1 68	Il1r2 69	Il1rn 70	Il2 71	Il20 72
Il22 73	Il2rb 74	Il2rg 75	Il3 76	Il4 77	Il5 78	Il5ra 79	Il6 80
Il6ra 81	Il6st 82	Il8ra 83	Il8rb 84	Il9 85	Il9r 86	Itgam 87	Itgb2 88
Lta 89	Ltb 90	Mif 91	Nos2 92	AI875142 93	Rac1 94	Scye1 95	Spp1 96
Tgfb1 97	Tlr1 98	Tlr2 99	Tlr3 100	Tlr4 101	Tlr5 102	Tlr6 103	Tlr7 104
Tlr8 105	Tlr9 106	Tnf 107	Tnfrsf1a 108	Tnfrsf1b 109	Tnfsf5 110	Tollip 111	Xcl1 112
Xcr1 113	Blank 114	PUC18 115	Blank 116	Blank 117	AS1R2 118	AS1R1 119	AS1 120
Rps27a 121	B2m 122	Hspcb 123	Hspcb 124	Ppia 125	Ppia 126	BAS2C 127	BAS2C

Microarray Gene Identification Information

Position	GeneBank	Symbol	Formal Name	Functional Importance
1	NM_008084	Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	Oxidoreductase activity;Mitochondrion;Glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) activity;Glucose metabolism;Glycolysis;Glyceraldehyde-3-phosphate dehydrogenase activity;
2	NM_007551	Blr1	Burkitt lymphoma receptor 1	Integral to membrane;G-protein coupled receptor protein signaling pathway;Receptor activity;Integral to plasma membrane;Rhodopsin-like receptor activity;Lymph gland development;B-cell activation;C-X-C chemokine receptor activity;Angiotensin type II receptor activity;G-protein coupled receptor activity;Purinergic nucleotide receptor activity, G-protein coupled;
3	NM_009778	C3	Complement component 3	Extracellular;Protein binding;Inflammatory response;Extracellular space;Endopeptidase inhibitor activity;Complement activation, alternative pathway;Complement activation, classical pathway;Complement activation;Positive regulation of phagocytosis;Positive regulation of type IIa hypersensitivity;
4	NM_011329	Ccl1	Expressed sequence BF534335	Signal transduction;Extracellular;Immune response;Protein binding;Extracellular space;Chemokine activity;Chemotaxis;Cytokine activity;
5	NM_011330	Ccl11	Small chemokine (C-C motif) ligand 11	Signal transduction;Extracellular;Immune response;Inflammatory response;Extracellular space;Chemokine activity;Chemotaxis;Cytokine activity;
6	NM_011331	Ccl12	Chemokine (C-C motif) ligand 12	Signal transduction;Extracellular;Immune response;Inflammatory response;Extracellular space;Chemokine activity;Chemotaxis;Cytokine activity;
7	NM_011332	Ccl17	Chemokine (C-C motif) ligand 17	Signal transduction;Extracellular;Immune response;Extracellular space;Chemokine activity;Chemotaxis;Cytokine activity;Cellular defense response;
8	NM_011888	Ccl19	Chemokine (C-C motif) ligand 19	Extracellular;Immune response;Inflammatory response;Extracellular space;Chemokine activity;Chemotaxis;Cytokine activity;
9	NM_011333	Ccl2	Chemokine (C-C motif) ligand 2	Signal transduction;Extracellular;Immune response;Protein binding;Inflammatory response;Extracellular space;Chemokine activity;Chemotaxis;Cytokine activity;G-protein-coupled receptor binding;

10	NM_016960	Ccl20	Chemokine (C-C motif) ligand 20	Signal transduction;Extracellular;Immune response;Protein binding;Inflammatory response;Extracellular space;Chemokine activity;Chemotaxis;Cytokine activity;
11	NM_011335	Ccl21a	Chemokine (C-C motif) ligand 21c (leucine)	Extracellular;Immune response;Inflammatory response;Chemokine activity;Chemotaxis;Cytokine activity;Immune cell chemotaxis;Negative regulation of myeloid blood cell differentiation;
12	NM_009137	Ccl22	Chemokine (C-C motif) ligand 22	Signal transduction;Extracellular;Immune response;Inflammatory response;Extracellular space;Chemokine activity;Chemotaxis;Cytokine activity;
13	NM_019577	Ccl24	Chemokine (C-C motif) ligand 24	Extracellular;Immune response;Inflammatory response;Extracellular space;Chemokine activity;Chemotaxis;Cytokine activity;
14	NM_009138	Ccl25	Chemokine (C-C motif) ligand 25	Signal transduction;Extracellular;Immune response;Protein binding;Inflammatory response;Extracellular space;Chemokine activity;Chemotaxis;Cytokine activity;
15	NM_011337	Ccl3	Chemokine (C-C motif) ligand 3	Signal transduction;Extracellular;Immune response;Protein binding;Inflammatory response;Extracellular space;Chemokine activity;Chemotaxis;Cytokine activity;
16	NM_013652	Ccl4	Chemokine (C-C motif) ligand 4	Signal transduction;Extracellular;Immune response;Inflammatory response;Extracellular space;Chemokine activity;Chemotaxis;Cytokine activity;
17	NM_013653	Ccl5	Chemokine (C-C motif) ligand 5	Signal transduction;Extracellular;Immune response;Inflammatory response;Extracellular space;Chemokine activity;Chemotaxis;Cytokine activity;
18	NM_009139	Ccl6	Chemokine (C-C motif) ligand 6	Signal transduction;Extracellular;Immune response;Extracellular space;Chemokine activity;Chemotaxis;Cytokine activity;
19	NM_013654	Ccl7	Chemokine (C-C motif) ligand 7	Signal transduction;Extracellular;Immune response;Inflammatory response;Receptor activity;Membrane;Extracellular space;Heparin binding;Chemokine activity;Chemotaxis;Transporter activity;Transport;Cytokine activity;
20	NM_021443	Ccl8	Chemokine (C-C motif) ligand 8	Signal transduction;Extracellular;Immune response;Inflammatory response;Extracellular space;Heparin binding;Chemokine activity;Chemotaxis;Cytokine activity;
21	NM_011338	Ccl9	Chemokine (C-C motif) ligand 9	Signal transduction;Extracellular;Immune response;Extracellular space;Chemokine activity;Chemotaxis;Cytokine activity;

22	NM_009912	Ccr1	Chemokine (C-C motif) receptor 1	Integral to membrane;Protein binding;Inflammatory response;G-protein coupled receptor protein signaling pathway;Receptor activity;Rhodopsin-like receptor activity;Immune cell chemotaxis;C-C chemokine receptor activity;G-protein coupled receptor activity;Neuropeptide Y receptor activity;Purinergic nucleotide receptor activity, G-protein coupled;Myeloid blood cell differentiation;
23	NM_009915	Ccr2	Chemokine (C-C motif) receptor 2	Integral to membrane;Immune response;Protein binding;Humoral immune response;Inflammatory response;G-protein coupled receptor protein signaling pathway;Chemotaxis;Defense response;Rhodopsin-like receptor activity;Hemopoiesis;C-C chemokine receptor activity;G-protein coupled receptor activity;Cytokine binding;Cellular defense response (sensu Vertebrata);Perception of pain;Regulation of cell migration;
24	NM_009914	Ccr3	Chemokine (C-C motif) receptor 3	Integral to membrane;Protein binding;G-protein coupled receptor protein signaling pathway;Receptor activity;Chemotaxis;Defense response;Rhodopsin-like receptor activity;C-C chemokine receptor activity;G-protein coupled receptor activity;Neuropeptide Y receptor activity;Purinergic nucleotide receptor activity, G-protein coupled;
25	NM_009916	Ccr4	Chemokine (C-C motif) receptor 4	Integral to membrane;Inflammatory response;G-protein coupled receptor protein signaling pathway;Chemotaxis;Rhodopsin-like receptor activity;C-C chemokine receptor activity;Angiotensin type II receptor activity;G-protein coupled receptor activity;
26	NM_009917	Ccr5	Chemokine (C-C motif) receptor 5	Integral to membrane;G-protein coupled receptor protein signaling pathway;Chemotaxis;Defense response;Rhodopsin-like receptor activity;C-C chemokine receptor activity;Cell surface;G-protein coupled receptor activity;
27	NM_009835	Ccr6	Chemokine (C-C motif) receptor 6	Integral to membrane;Protein binding;G-protein coupled receptor protein signaling pathway;Receptor activity;Rhodopsin-like receptor activity;C-C chemokine receptor activity;G-protein coupled receptor activity;
28	NM_007719	Ccr7	Chemokine (C-C motif) receptor 7	Integral to membrane;Immune response;G-protein coupled receptor protein signaling pathway;Receptor activity;Extracellular space;Chemotaxis;Rhodopsin-like receptor activity;C-C chemokine receptor activity;G-protein coupled receptor activity;

29	NM_007720	Ccr8	Chemokine (C-C motif) receptor 8	Integral to membrane;Protein binding;G-protein coupled receptor protein signaling pathway;Chemotaxis;Defense response;Rhodopsin-like receptor activity;C-C chemokine receptor activity;G-protein coupled receptor activity;
30	NM_009913	Ccr9	Chemokine (C-C motif) receptor 9	Integral to membrane;Protein binding;G-protein coupled receptor protein signaling pathway;Receptor activity;Chemotaxis;Defense response;Rhodopsin-like receptor activity;C-C chemokine receptor activity;G-protein coupled receptor activity;
31	NM_009142	Cx3cl1	Chemokine (C-X3-C motif) ligand 1	Integral to membrane;Cell adhesion;Signal transduction;Extracellular;Immune response;Protein binding;Membrane;Chemokine activity;Cytokine activity;
32	NM_009987	Cx3cr1	Chemokine (C-X3-C) receptor 1	Integral to membrane;G-protein coupled receptor protein signaling pathway;Receptor activity;Rhodopsin-like receptor activity;C-C chemokine receptor activity;Chemokine receptor activity;G-protein coupled receptor activity;
33	NM_008176	Cxcl1	Chemokine (C-X-C motif) ligand 1	Extracellular;Immune response;Cell growth and/or maintenance;Regulation of cell cycle;Inflammatory response;Intracellular;Extracellular space;Growth factor activity;Chemokine activity;Cytokine activity;
34	NM_021274	Cxcl10	Chemokine (C-X-C motif) ligand 10	Extracellular;Immune response;Inflammatory response;Extracellular space;Chemokine activity;Chemotaxis;Cytokine activity;
35	NM_019494	Cxcl11	Chemokine (C-X-C motif) ligand 11	Extracellular;Immune response;Inflammatory response;Extracellular space;Chemokine activity;Chemotaxis;Cytokine activity;
36	NM_021704	Cxcl12	Chemokine (C-X-C motif) ligand 12	Extracellular;Immune response;Germ cell development;Extracellular space;Growth factor activity;Chemokine activity;Chemotaxis;Cytokine activity;Regulation of cell migration;Brain development;T-cell proliferation;Positive regulation of cell migration;Induction of positive chemotaxis;Germ cell migration;
37	NM_018866	Cxcl13	Chemokine (C-X-C motif) ligand 13	Extracellular;Immune response;Inflammatory response;Chemokine activity;Chemotaxis;Cytokine activity;Lymph gland development;
38	NM_019568	Cxcl14	Chemokine (C-X-C motif) ligand 14	Extracellular;Immune response;Extracellular space;Chemokine activity;Cytokine activity;

39	NM_011339	Cxcl15	Chemokine (C-X-C motif) ligand 15	Signal transduction;Extracellular;Immune response;Inflammatory response;Extracellular space;Chemokine activity;Chemotaxis;Cytokine activity;Hemopoiesis;Neutrophil chemotaxis;
40	NM_009140	Cxcl2	Chemokine (C-X-C motif) ligand 2	Signal transduction;Extracellular;Immune response;Inflammatory response;Chemokine activity;Chemotaxis;Cytokine activity;
41	NM_019932	Cxcl4	Chemokine (C-X-C motif) ligand 4	Extracellular;Immune response;Extracellular space;Heparin binding;Chemokine activity;Chemotaxis;Cytokine activity;
42	NM_009141	Cxcl5	Chemokine (C-X-C motif) ligand 5	Signal transduction;Extracellular;Immune response;Inflammatory response;Extracellular space;Chemokine activity;Chemotaxis;Cytokine activity;
43	NM_008599	Cxcl9	Chemokine (C-X-C motif) ligand 9	Extracellular;Immune response;Inflammatory response;Extracellular space;Chemokine activity;Cytokine activity;
44	NM_009910	Cxcr3	Chemokine (C-X-C motif) receptor 3	Integral to membrane;G-protein coupled receptor protein signaling pathway;Receptor activity;Chemotaxis;Defense response;Rhodopsin-like receptor activity;C-C chemokine receptor activity;Chemokine receptor activity;C-X-C chemokine receptor activity;G-protein coupled receptor activity;Purinergic nucleotide receptor activity, G-protein coupled;
45	NM_010185	Fcer1g	Fc receptor, IgE, high affinity I, gamma polypeptide	Plasma membrane;Integral to membrane;Signal transduction;Cell surface receptor linked signal transduction;Receptor activity;Membrane;Integral to plasma membrane;Transmembrane receptor activity;Humoral defense mechanism (sensu Vertebrata);Neutrophil chemotaxis;Regulation of immune response;Antigen presentation, exogenous antigen via MHC class II;External side of plasma membrane;Positive regulation of tumor necrosis factor-alpha biosynthesis;Lipid raft;Positive regulation of phagocytosis;Positive regulation of type IIa hypersensitivity;IgE binding;IgG binding;Positive regulation of interleukin-10 biosynthesis;Positive regulation of immune response;Antigen presentation, exogenous antigen via MHC class I;Defense response to pathogenic bacteria;Phagocytosis, engulfment;Positive regulation of type III hypersensitivity;

46	NM_010186	Fcgr1	Fc receptor, IgG, high affinity I	Integral to membrane;Cell surface receptor linked signal transduction;Receptor activity;Extracellular space;Receptor mediated endocytosis;Regulation of immune response;External side of plasma membrane;Positive regulation of phagocytosis;Positive regulation of type IIa hypersensitivity;Antigen presentation, exogenous antigen;IgG binding;IgG receptor activity;Antibody-dependent cellular cytotoxicity;Antigen presentation, exogenous antigen via MHC class I;Defense response to pathogenic bacteria;Phagocytosis, engulfment;Phagocytosis, recognition;Positive regulation of type III hypersensitivity;
47	NM_007721	Gpr2	G protein-coupled receptor 2	Integral to membrane;Protein binding;G-protein coupled receptor protein signaling pathway;Receptor activity;Integral to plasma membrane;Chemotaxis;Defense response;Rhodopsin-like receptor activity;C-C chemokine receptor activity;Cytosolic calcium ion concentration elevation;Chemokine receptor activity;G-protein coupled receptor activity;
48	NM_010503	Ifna2	Interferon alpha family, gene 2	Extracellular;Extracellular space;Defense response;Cytokine activity;Hematopoietin/interferon-class (D200-domain) cytokine receptor binding;
49	NM_008337	Ifng	Interferon gamma	Regulation of cell growth;Extracellular;Immune response;Transcriptional activator activity;Extracellular space;Defense response;Regulation of transcription;Cytokine activity;Interferon-gamma receptor binding;Neutrophil chemotaxis;Positive regulation of transcription, DNA-dependent;Positive regulation of MHC class II biosynthesis;Positive regulation of isotype switching to IgG isotypes;Regulation of immune response;Programmed cell death, neutrophils;Positive regulation of interleukin-12 biosynthesis;Positive regulation of interleukin-6 biosynthesis;Positive regulation of chemokine biosynthesis;Positive regulation of interleukin-1 beta secretion;Programmed cell death, inflammatory cells;
50	XM_111360	Igh-4	Immunoglobulin heavy chain 4 (serum IgG1)	

51	NM_010548	Il10	Interleukin 10	Extracellular;Immune response;Extracellular space;Cytokine activity;Positive regulation of MHC class II biosynthesis;
52	NM_008348	Il10ra	Interleukin 10 receptor, alpha	Integral to membrane;Cell surface receptor linked signal transduction;Receptor activity;Membrane;Integral to plasma membrane;Extracellular space;Hematopoietin/interferon-class (D200-domain) cytokine receptor activity;Interleukin receptor activity;
53	NM_008349	Il10rb	Interleukin 10 receptor, beta	Integral to membrane;Protein binding;Cell surface receptor linked signal transduction;Receptor activity;Membrane;Integral to plasma membrane;Hematopoietin/interferon-class (D200-domain) cytokine receptor activity;Interleukin-10 receptor activity;
54	NM_008350	Il11	Interleukin 11	Extracellular space;Growth factor activity;Cytokine activity;
55	NM_008351	Il12a	Interleukin 12A	Extracellular;Immune response;Extracellular space;Growth factor activity;Cytokine activity;Interleukin-12 receptor binding;
56	NM_008352	Il12b	Interleukin 12B	Cell surface receptor linked signal transduction;Membrane;Extracellular space;Cytokine activity;Hematopoietin/interferon-class (D200-domain) cytokine receptor activity;Protein homodimerization activity;
57	NM_008354	Il12rb2	Interleukin 12 receptor, beta 2	Integral to membrane;Cell surface receptor linked signal transduction;Receptor activity;Membrane;Integral to plasma membrane;Extracellular space;Hematopoietin/interferon-class (D200-domain) cytokine receptor activity;Interleukin receptor activity;
58	NM_008355	Il13	Interleukin 13	Extracellular;Immune response;Extracellular space;Cytokine activity;Interleukin-13 receptor binding;Hematopoietin/interferon-class (D200-domain) cytokine receptor binding;
59	NM_133990	Il13ra1	Interleukin 13 receptor, alpha 1	Integral to membrane;Cell surface receptor linked signal transduction;Receptor activity;Membrane;Integral to plasma membrane;Extracellular space;Hematopoietin/interferon-class (D200-domain) cytokine receptor activity;Interleukin receptor activity;

60	NM_008357	Il15	Interleukin 15	Extracellular;Immune response;Cytokine activity;Lymph gland development;Hematopoietin/interferon-class (D200-domain) cytokine receptor binding;Regulation of T-cell differentiation;Positive regulation of T-cell proliferation;NK T-cell proliferation;Extrathymic T-cell selection;Positive regulation of immune response;Regulation of antiviral response by host;
61	NM_010551	Il16	Interleukin 16	Protein binding;Intracellular;Cytokine activity;Immune cell chemotaxis;Induction of positive chemotaxis;
62	NM_010552	Il17	Interleukin 17	Extracellular space;Cytokine activity;
63	NM_019508	Il17b	Interleukin 17B	Receptor binding;Protein binding;Extracellular space;Cytokine activity;Neutrophil chemotaxis;
64	NM_080729	Il17e	Interleukin 17E	Inflammatory response;Extracellular space;Cytokine activity;Response to pathogenic fungi;Interleukin-17E receptor binding;Eosinophil differentiation;Response to nematodes;
65	NM_008360	Il18	Interleukin 18	Extracellular;Immune response;Interleukin-1 receptor binding;Cytokine activity;
66	NM_010554	Il1a	Interleukin 1 alpha	Cell proliferation;Extracellular;Immune response;Regulation of cell cycle;Inflammatory response;Signal transducer activity;Growth factor activity;Interleukin-1 receptor binding;Cytokine and chemokine mediated signaling pathway;Cytokine activity;
67	NM_008361	Il1b	Interleukin 1 beta	Cell proliferation;Extracellular;Immune response;Regulation of cell cycle;Inflammatory response;Signal transducer activity;Growth factor activity;Interleukin-1 receptor binding;Cytokine and chemokine mediated signaling pathway;Cytokine activity;Neutrophil chemotaxis;Positive regulation of interleukin-6 biosynthesis;Positive regulation of chemokine biosynthesis;
68	NM_008362	Il1r1	Interleukin 1 receptor, type I	Integral to membrane;Signal transducer activity;Cell surface receptor linked signal transduction;Receptor activity;Membrane;Integral to plasma membrane;Extracellular space;Transmembrane receptor activity;Interleukin-1, Type I, activating receptor activity;Cytokine and chemokine mediated signaling pathway;Interleukin-1

				receptor activity;Interleukin receptor activity;
69	NM_010555	Il1r2	Interleukin 1 receptor, type II	ATP binding;Integral to membrane;Cell surface receptor linked signal transduction;Receptor activity;Membrane;Integral to plasma membrane;Extracellular space;Interleukin-1, Type II, blocking receptor activity;Transport;ATP-binding cassette (ABC) transporter activity;Interleukin-1 receptor activity;Interleukin receptor activity;
70	NM_031167	Il1rn	Interleukin 1 receptor antagonist	Extracellular;Immune response;Cell surface receptor linked signal transduction;Receptor activity;Integral to plasma membrane;Interleukin-1 receptor binding;Lipid metabolism;Insulin secretion;
71	NM_008366	Il2	Interleukin 2	Cell proliferation;Extracellular;Immune response;Inflammatory response;Extracellular space;Growth factor activity;Hormone activity;Defense response;Cytokine activity;Cellular defense response;Interleukin-2 receptor binding;
72	NM_021380	Il20	Interleukin 20	Extracellular;Immune response;Extracellular space;Cytokine activity;STAT protein nuclear translocation;
73	NM_016971	Il22	Interleukin 22	Extracellular;Immune response;Protein binding;Cytokine activity;Oxygen and reactive oxygen species metabolism;Regulation of tyrosine phosphorylation of Stat3 protein;
74	NM_008368	Il2rb	Interleukin 2 receptor, beta chain	Integral to membrane;Cell surface receptor linked signal transduction;Receptor activity;Membrane;Integral to plasma membrane;Extracellular space;Hematopoietin/interferon-class (D200-domain) cytokine receptor activity;Interleukin receptor activity;
75	NM_013563	Il2rg	Interleukin 2 receptor, gamma chain	Integral to membrane;Cell surface receptor linked signal transduction;Receptor activity;Membrane;Integral to plasma membrane;Extracellular space;Hematopoietin/interferon-class (D200-domain) cytokine receptor activity;Interleukin receptor activity;
76	NM_010556	Il3	Interleukin 3	Extracellular;Immune response;Extracellular space;Growth factor activity;Cytokine activity;Interleukin-3 receptor binding;
77	NM_021283	Il4	Interleukin 4	Extracellular;Immune response;Extracellular space;Growth factor activity;Cytokine activity;Interleukin-4 receptor binding;Hematopoietin/interferon-class (D200-domain) cytokine receptor binding;B-cell activation;Negative regulation of

				osteoclast differentiation;Positive regulation of MHC class II biosynthesis;Positive regulation of isotype switching to IgG isotypes;Regulation of immune response;Regulation of phosphorylation;
78	NM_010558	Il5	Interleukin 5	Extracellular;Immune response;Extracellular space;Growth factor activity;Cytokine activity;Interleukin-5 receptor binding;
79	NM_008370	Il5ra	Interleukin 5 receptor, alpha	Integral to membrane;Cell surface receptor linked signal transduction;Receptor activity;Membrane;Integral to plasma membrane;Extracellular space;Hematopoietin/interferon-class (D200-domain) cytokine receptor activity;Interleukin receptor activity;
80	NM_031168	Il6	Interleukin 6	Extracellular;Immune response;Protein binding;Extracellular space;Acute-phase response;Growth factor activity;Cytokine activity;Interleukin-6 receptor binding;Negative regulation of chemokine biosynthesis;Programmed cell death, neutrophils;
81	NM_010559	Il6ra	Interleukin 6 receptor, alpha	Integral to membrane;Cell surface receptor linked signal transduction;Receptor activity;Membrane;Integral to plasma membrane;Extracellular space;Hematopoietin/interferon-class (D200-domain) cytokine receptor activity;Interleukin receptor activity;
82	NM_010560	Il6st	Interleukin 6 signal transducer	Integral to membrane;Signal transduction;Receptor activity;Membrane;Extracellular space;Hematopoietin/interferon-class (D200-domain) cytokine receptor activity;Regulation of Notch signaling pathway;
83	NM_178241	Il8ra	Interleukin 8 receptor, alpha	Integral to membrane;G-protein coupled receptor protein signaling pathway;Receptor activity;G-protein coupled receptor activity;
84	NM_009909	Il8rb	Interleukin 8 receptor, beta	Integral to membrane;G-protein coupled receptor protein signaling pathway;Receptor activity;Integral to plasma membrane;Chemotaxis;Defense response;Rhodopsin-like receptor activity;Interleukin-8 receptor activity;Chemokine receptor activity;G-protein coupled receptor activity;
85	NM_008373	Il9	Interleukin 9	Extracellular;Immune response;Extracellular space;Growth factor activity;Cytokine activity;Interleukin-9 receptor binding;Hematopoietin/interferon-class (D200-domain) cytokine receptor binding;
86	NM_008374	Il9r	Interleukin 9	Integral to membrane;Cell surface receptor

			receptor	linked signal transduction;Receptor activity;Membrane;Integral to plasma membrane;Hematopoietin/interferon-class (D200-domain) cytokine receptor activity;Interleukin receptor activity;
87	NM_008401	Itgam	Integrin alpha M	Integral to membrane;Cell adhesion;Protein binding;Receptor activity;Cell-matrix adhesion;Integrin-mediated signaling pathway;Integrin complex;Neutrophil chemotaxis;Cellular extravasation;External side of plasma membrane;Opsonin binding;
88	NM_008404	Itgb2	Integrin beta 2	Integral to membrane;Cell adhesion;Protein binding;Development;Receptor activity;Cell-matrix adhesion;Extracellular space;Integrin-mediated signaling pathway;Integrin complex;Neutrophil chemotaxis;Cellular extravasation;
89	NM_010735	Lta	Lymphotoxin A	Cell proliferation;Cell growth and/or maintenance;Humoral immune response;Inflammatory response;Positive regulation of cell proliferation;Extracellular space;Tumor necrosis factor receptor binding;Cellular defense response;Lymph gland development;Programmed cell death, transformed cells;
90	NM_008518	Ltb	Lymphotoxin B	Plasma membrane;Integral to membrane;Lymph gland development;
91	NM_010798	Mif	Macrophage migration inhibitory factor	Inflammatory response;Cell aging;Regulation of cell proliferation;Cytokine activity;Isomerase activity;DNA damage response, signal transduction by p53 class mediator;
92	NM_010927	Nos2	Nitric oxide synthase 2, inducible, macrophage	Electron transporter activity;Oxidoreductase activity;Nitric oxide biosynthesis;Electron transport;Superoxide metabolism;Calmodulin binding;Nitric-oxide synthase activity;NOT ovulation (sensu Mammalia);Defense response to bacteria;FMN binding;
93	NM_011101	AI875142	Expressed sequence AI875142	ATP binding;Transferase activity;Protein amino acid phosphorylation;Calcium ion binding;Nucleus;Protein binding;Cytoplasm;Diacylglycerol binding;Protein serine/threonine kinase activity;Intracellular signaling cascade;Protein-tyrosine kinase activity;Protein kinase activity;Induction of apoptosis by intracellular signals;Negative regulation of protein kinase activity;Neutrophil chemotaxis;Regulation of peptidyl-tyrosine phosphorylation;Positive regulation of inflammatory

				response;Negative regulation of glucose import;Protein kinase C activity;Calcium-dependent protein kinase C activity;Induction of positive chemotaxis;Inactivation of MAPK;Negative regulation of insulin receptor signaling pathway;
94	NM_009007	Rac1	RAS-related C3 botulinum substrate 1	GTP binding;GTPase activity;Small GTPase mediated signal transduction;Cell adhesion;Protein binding;Cytoplasm;Intracellular signaling cascade;Inflammatory response;Response to wounding;Cell motility;Endocytosis;Dendrite morphogenesis;Cytoplasmic vesicle;Positive regulation of actin filament polymerization;Extrinsic to plasma membrane;Lamellipodium;Hyperosmotic response;Lamellipodium biogenesis;
95	NM_007926	Scye1	Small inducible cytokine subfamily E, member 1	Nucleic acid binding;Cytokine activity;TRNA binding;Protein biosynthesis;RNA binding;
96	NM_009263	Spp1	Secreted phosphoprotein 1	Cell adhesion;Protein binding;Extracellular space;Cytokine activity;Ossification;
97	NM_011577	Tgfb1	Transforming growth factor, beta 1	Protein amino acid phosphorylation;Cell proliferation;Protein binding;Negative regulation of cell proliferation;Regulation of cell cycle;Inflammatory response;Extracellular matrix;Extracellular space;Organogenesis;Growth factor activity;Skeletal development;Defense response;Regulation of cell proliferation;Necrosis;Transforming growth factor beta receptor signaling pathway;Transforming growth factor beta receptor binding;Cell growth;Lymph gland development;Growth;Myogenesis;Regulation of myogenesis;Regulation of protein-nucleus import;
98	NM_030682	Tlr1	Toll-like receptor 1	Integral to membrane;Immune response;Inflammatory response;Receptor activity;Membrane;Transmembrane receptor activity;
99	NM_011905	Tlr2	Toll-like receptor 2	Plasma membrane;Integral to membrane;Immune response;Inflammatory response;Cell surface receptor linked signal transduction;Receptor activity;Membrane;Transmembrane receptor activity;Positive regulation of tumor necrosis factor-alpha biosynthesis;
100	NM_126166	Tlr3	Toll-like receptor 3	Integral to membrane;Immune response;Inflammatory response;Response

				to virus;Receptor activity;Membrane;Extracellular space;Transmembrane receptor activity;Defense response;
101	NM_021297	Tlr4	Toll-like receptor 4	Integral to membrane;Immune response;Inflammatory response;Receptor activity;Membrane;Extracellular space;Transmembrane receptor activity;Metabolism;I-kappaB kinase/NF-kappaB cascade;Catalytic activity;Toll signaling pathway;
102	NM_016928	Tlr5	Toll-like receptor 5	Nucleic acid binding;Integral to membrane;Immune response;Inflammatory response;Receptor activity;Membrane;Extracellular space;Transmembrane receptor activity;Actin binding;
103	NM_011604	Tlr6	Toll-like receptor 6	Integral to membrane;Immune response;Inflammatory response;Receptor activity;Membrane;Transmembrane receptor activity;
104	NM_133211	Tlr7	Toll-like receptor 7	Integral to membrane;Immune response;Inflammatory response;Receptor activity;Membrane;Extracellular space;Transmembrane receptor activity;
105	NM_133212	Tlr8	Toll-like receptor 8	Integral to membrane;Immune response;Inflammatory response;Receptor activity;Membrane;Extracellular space;Transmembrane receptor activity;
106	NM_031178	Tlr9	Toll-like receptor 9	Plasma membrane;Integral to membrane;Immune response;Inflammatory response;Response to virus;Receptor activity;Membrane;Extracellular space;Transmembrane receptor activity;
107	NM_013693	Tnf	Tumor necrosis factor	Plasma membrane;Cell proliferation;Immune response;Cell growth and/or maintenance;Humoral immune response;Inflammatory response;Positive regulation of cell proliferation;Induction of apoptosis via death domain receptors;Signal transducer activity;Development;Positive regulation of I-kappaB kinase/NF-kappaB cascade;Integral to plasma membrane;Organogenesis;Tumor necrosis factor receptor binding;Defense response;Secretory granule;Regulation of cell proliferation;Cytokine and chemokine mediated signaling pathway;Cellular defense response;Lymph gland development;Cellular extravasation;Positive regulation of osteoclast differentiation;Programmed cell death, transformed cells;Regulation of

				osteoclast differentiation;
108	NM_011609	Tnfrsf1a	Tumor necrosis factor receptor superfamily, member 1a	Plasma membrane;Integral to membrane;Signal transduction;Protein binding;Intracellular signaling cascade;Apoptosis;Inflammatory response;Cell surface receptor linked signal transduction;Receptor activity;Extracellular space;Cell death;Tumor necrosis factor receptor binding;Defense response;Tumor necrosis factor receptor activity;Lymph gland development;Prostaglandin metabolism;
109	NM_011610	Tnfrsf1b	Tumor necrosis factor receptor superfamily, member 1b	Integral to membrane;Cell proliferation;Inflammatory response;Cell surface receptor linked signal transduction;Receptor activity;Extracellular space;Necrosis;
110	NM_011616	Tnfsf5	Tumor necrosis factor (ligand) superfamily, member 5	Plasma membrane;Integral to membrane;Immune response;Membrane;Tumor necrosis factor receptor binding;Defense response;Cytokine activity;
111	NM_023764	Tollip	Toll interacting protein	Immune response;Inflammatory response;Signal transducer activity;
112	NM_008510	Xcl1	Chemokine (C motif) ligand 1	Extracellular;Immune response;Extracellular space;Chemokine activity;Chemotaxis;Cytokine activity;
113	NM_011798	Xcr1	Chemokine (C motif) receptor 1	Integral to membrane;G-protein coupled receptor protein signaling pathway;Receptor activity;Rhodopsin-like receptor activity;Chemokine receptor activity;G-protein coupled receptor activity;
114				
115	L08752	PUC18	PUC18 Plasmid DNA	
116				
117				
118	SA_00005	AS1R2	Artificial Sequence 1 Related 2 (80% identity)(48/60)	
119	SA_00004	AS1R1	Artificial Sequence 1 Related 1 (90% identity)(54/60)	
120	SA_00003	AS1	Artificial Sequence 1	
121	NM_024277	Rps27a	Ribosomal protein S27a	Intracellular;Protein biosynthesis;Ribosome;
122	NM_009735	B2m	Beta-2 microglobulin	Plasma membrane;Integral to plasma membrane;Extracellular space;Defense response;Cellular defense response;Antigen processing, endogenous antigen via MHC class I;MHC class I receptor activity;Antigen presentation, endogenous antigen;
123	NM_008302	Hspcb	Heat shock protein	ATP binding;Protein

			1, beta	binding;Mitochondrion;Response to heat;Protein folding;Unfolded protein binding;Response to unfolded protein;ATP binding;Protein binding;Mitochondrion;Response to heat;Protein folding;Unfolded protein binding;Response to unfolded protein;
124	NM_008302	Hspcb	Heat shock protein 1, beta	ATP binding;Protein binding;Mitochondrion;Response to heat;Protein folding;Unfolded protein binding;Response to unfolded protein;ATP binding;Protein binding;Mitochondrion;Response to heat;Protein folding;Unfolded protein binding;Response to unfolded protein;
125	NM_008907	Ppia	Peptidylprolyl isomerase A	Cytosol;Protein folding;Isomerase activity;Peptidyl-prolyl cis-trans isomerase activity;Cytosol;Protein folding;Isomerase activity;Peptidyl-prolyl cis-trans isomerase activity;
126	NM_008907	Ppia	Peptidylprolyl isomerase A	Cytosol;Protein folding;Isomerase activity;Peptidyl-prolyl cis-trans isomerase activity;Cytosol;Protein folding;Isomerase activity;Peptidyl-prolyl cis-trans isomerase activity;
127	SA_00007	BAS2C	Biotinylated Artificial Sequence 2 Complementary sequence	
128	SA_00007	BAS2C	Biotinylated Artificial Sequence 2 Complementary sequence	

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BIOGRAPHICAL SKETCH

Kelli Herrlinger-Garcia was born in Bloomington, Indiana, and moved to Florida in 1973. She graduated from Gainesville High School in 1989. Kelli earned an Associate of Arts degree in the summer of 1991 from Santa Fe Community College, and graduated with highest honors in May of 1993 with a Bachelor of Science in interdisciplinary studies with a concentration in biochemistry and molecular biology. Kelli worked for two years as a scientist for the Shriner's Childrens Hospital-Tampa Unit. Kelli then began work for Dr. Bobbi Langkamp-Henken in the Food Science and Human Nutrition Department at the University of Florida, where she has been employed for more than 10 years. Kelli began the Master of Science program in the fall of 2003, while continuing to work full-time with Dr. Bobbi Langkamp-Henken. Kelli's achievements include publication of 27 original research works and abstracts, and she was a 1997 University of Florida Superior Accomplishment Award winner. During her graduate education she received the William and Agnes Brown Scholarship; was nominated to Gamma Sigma Delta: The Honor Society of Agriculture; and was an American Society of Nutrition Procter & Gamble Graduate Student Competition Abstract Winner. In addition to her work, Kelli has a son, Austin, whom she coaches in YSI Incorporated and the City of Gainesville soccer teams. Kelli will be awarded a Master of Science degree in food science and human nutrition with a specialization in nutritional science in August of 2006.