

ANTI-INFLAMMATORY IMMUNE COMPETENCE IN THE ACUTELY  
MALNOURISHED WEANLING MOUSE

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## **ABSTRACT**

### **ANTI-INFLAMMATORY IMMUNE COMPETENCE IN THE ACUTELY MALNOURISHED WEANLING MOUSE**

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This thesis is an investigation centered on identifying the potential underlying contributors toward the classically depressed inflammatory cell-mediated form of immune competence in acute (i.e., wasting) protein energy malnutrition (PEM). Two metabolically distinct weight loss pathologies, marasmus (energy deficit) and incipient kwashiorkor (dietary protein deficit), which mimic the critical features of the human condition were reproduced in weanling C57BL/6J mice. In the advanced stages of carcass energy losses we demonstrated the dominance of a type-2 polarizing influence within the effector/memory T cell compartment, wherein systemic production of interferon(IFN)- $\gamma$  was depressed and interleukin(IL)-4 production was sustained. The hormonal response to wasting pathology, which exerts a permissive influence on the aforementioned polarization phenomenon, prompted an investigation into the anti-inflammatory and tolerogenic form of immune competence. Using appropriately crafted assay strategies, blood levels of a triad of hormones glucocorticoids (GC), transforming growth factor(TGF)- $\beta$  and IL-10 were determined to rise early and remain elevated despite advancing weight loss.

Thus, this hormonal triad is positioned temporally to act in both an initiating and sustaining capacity in acute malnutrition. Mechanistically, the elevated blood IL-10 levels in acute PEM are likely attributed to new cytokine production, as evidenced by increased or sustained levels of mRNA expression in lymphoid organs and a systemic cytokine protein production rate that is at least sustained throughout the duration of wasting pathology. Anti-inflammatory immune competence consists of tiers, wherein the aforementioned outcomes represent a primary tier. The capacity to produce IL-10 above constitutive levels, i.e., second tier responses to stimuli, in the innate and combined innate and adaptive arms of the immune system were depressed in the advanced stages of weight loss wherein carcass energy losses were the most pronounced. Overall, the outcomes of this thesis provide the experimental basis supporting the proposition that malnutrition-associated immune depression represents a regulated pathophysiology wherein the anti-inflammatory and tolerogenic form of immune competence is preserved and likely to play an initiating role in inflammatory immune depression. A new model emerges wherein the adaptive benefit conferred via a triad of anti-inflammatory hormones is the active suppression of inflammatory immune responses generated against catabolically released self-antigens.

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## **Chapter One**

### **Literature Review**

#### **1. Protein-Energy Malnutrition**

Protein-energy malnutrition (PEM) manifests in either an acute (i.e. wasting) or chronic (i.e. stunting) form resulting in altered body composition and reduced linear growth, respectively [1]. Globally, malnutrition represents a major public health problem and is a substantive underlying factor in over 50% of the 10-11 million deaths occurring in children under five years of age who die annually from preventable causes [2]. In infants and children, acute PEM is identified based on reduced weight-for-height (w/h) compared to standards for North American and European children and is graded according to degrees of perceived severity, i.e., grade I (80-90% w/h), grade II (70-80% w/h) and grade III (<70% w/h) [1]. The risk of mortality in acute malnutrition is directly related to severity [2] where the most severe form of acute (i.e., wasting) PEM, grade III, represents an end-stage disease that manifests in three clinical forms namely, marasmus, kwashiorkor and the combined form, marasmic-kwashiorkor [3]. This thesis is centered on grade III wasting malnutrition in the metabolically distinct end-stage forms of marasmus and kwashiorkor.

The dietary characteristic associated with marasmus is a low intake of all nutrients and thus, energy, and represents a condition of extreme emaciation wherein adipose and muscle stores are depleted in order to sustain essential visceral functions [3]. This metabolic form of wasting malnutrition differs from kwashiorkor, which results from dietary imbalance, particularly a low percentage of calories derived from protein. The kwashiorkor patient exhibits a less complete mobilization of subcutaneous fat relative to

the marasmic condition and is further characterized by the development of edema and hepatic pathology identified clinically by a palpable fatty liver [3,4].

## **2. Malnutrition and Susceptibility to Infection**

A cyclic relationship exists between acute malnutrition and infection susceptibility. This relationship is centered on the contribution of malnutrition-associated immune depression which is extensively documented in the early pre-pubescent stage of life [3,5-8]. In this connection, infection-related immune depression is a determinative risk factor in the infection-related morbidity and mortality of PEM [3]. In severely malnourished patients both acquired and innate immune defences are simultaneously diminished [3,8,9]. Malnutrition-associated infections are caused most commonly by organisms that are normally harmless commensals, namely opportunistic respiratory and intestinal infections, and the risk of mortality following infection with pathogenic organisms can be increased dramatically by nutritional deficit [8,9]. Severely malnourished patients will succumb to infections despite vigorous medical interventions [10], as fatality rates among hospitalized patients in developing countries is between 20-30% for marasmic patients and 50-60% for kwashiorkor patients [2]. Treatment strategies for the malnourished patient must first address the associated problems of sepsis or severe trauma and, without doing so, reversing the wasting process is rarely achievable [10].

### **3. Overview of Immune Defences**

#### **3.1 Innate Immunity**

Immune defences are divided into two broad components, innate and adaptive. Innate immunity is less specific and, through multiple mechanisms, provides the first line of defence against infection [11]. Defensive barriers employed by the innate immune system include anatomical barriers, such as the skin and mucosal membranes lining the gastro-intestinal tract, which prevent entry of most microorganisms. Additionally, soluble proteins in the blood and/or tissue fluids contribute to innate immunity. These include hydrolytic enzymes such as lysozyme which cleaves peptidoglycan in bacterial cell walls, the antiviral properties of interferons and complement proteins which target and promote the clearance of pathogenic organisms [11]. Further, phagocytic cells ingest foreign extracellular particulate material (i.e., infectious agents, dead cells and other debris) and many of these cells participate in inflammatory responses generated against tissue damage by a wound or by an invading pathogenic micororganism [11].

#### **3.2 Adaptive Immunity**

The adaptive immune system recognizes and selectively eliminates specific foreign microorganisms and molecules (i.e., foreign antigens). Antigenic specificity permits the adaptive immune system to distinguish subtle differences among antigens, thereby providing the capability to recognize and generate responses against a tremendous diversity of foreign antigen structures [11]. Additionally, the adaptive immune system exhibits the characteristic of immunological memory wherein, upon re-encountering an antigen, a more rapid and intense response is generated compared to the

initial response [11]. Lastly, adaptive immune responses are normally generated against foreign antigens; therefore the cells have the ability to distinguish between self and non-self cells. There are two arms of the adaptive immune system, humoral and cell-mediated, and the effectors of these types of responses are antibodies and activated T cell subsets, respectively.

### **3.3 Type-1 versus Type-2 Polarization of Immune Responses**

Adaptive immune responses manifest in one of two basic forms: a cell-mediated immune response or an antibody response. Activated CD4<sup>+</sup> T helper (Th) cells produce cytokines which direct immune responses toward one or the other of these polarized endpoints. Type-1 cytokines (IFN- $\gamma$ , IL-2 and TNF- $\beta$ ) preferentially induce cellular responses characterized by local inflammation and activation of cytotoxic T cells, both of which are required for the clearance of intracellular pathogens [12]. Additionally, they promote opsonizing and complement-fixing subclasses of IgG antibodies that also participate in defence against intracellular pathogens [11,13]. Interferon(IFN)- $\gamma$  is the signature type-1 polarizing cytokine, primarily directing acquired immune responses in a cell-mediated direction (i.e., manifesting in delayed-type hypersensitivity reactions and activation of cytotoxic T cells) [13,14]. Type-1 polarization is further influenced by IL-12, which is a central inducer of cell-mediated immunity by promoting the development, proliferation and function of Th1 cells [15,16]. Interleukin(IL)-4 is the signature type-2 polarizing cytokine which promotes the development of antibody responses [13,14]. Other cytokines can be involved in the promotion of type-2 responses, namely IL-5,-6, -10 and -13 [13,14], contributing toward the generation of an antibody response and

ultimately the clearance of extracellular pathogens. IL-4 is crucial for the production of immunoglobulins (Ig) by B cells, whereas IL-5 supports eosinophil development and activation [12]. Overall, effective clearance of a pathogen relies on antigen-specific Th cells with an adequate type-1 or type-2 cytokine production profile [12].

The polarizing cytokines produced by CD4<sup>+</sup> Th1 (IFN- $\gamma$ ) and Th2 (IL-4) effector cell populations have two characteristic effects on subsequent Th subset development. First, they promote the growth of the subset that produces them; second, they inhibit the development and activity of the opposite subset [11]. Thus type-1 and type-2 polarized cytokine responses are cross-regulated and mutually inhibitory.

Although type-1 and type-2 polarization is best described in the Th effector cell population, other immunological cell types also exhibit immunological polarization. In this connection, the antigen-presenting cell (APC) capable of stimulating naïve T cells, the dendritic cell (DC) [17], exhibits Th1-inducing and Th2-inducing DC subtypes, DC1 and DC2 respectively, at least in vitro [13,18,19]. Other immunological cells also exhibit either a type-1 (inflammatory) or type-2 (non-inflammatory) mature phenotype and cytokine production profile, e.g., macrophages [20-22], B lymphocytes [23] and natural killer cells [24].

## **4. Regulatory T Cells and Mechanisms of Immunological Tolerance**

### **4.1 Immunological Tolerance**

Tolerance to self-antigens is an active process that has both central and peripheral components. The redundancy of these components ensures that self-reactive T cells which escape detection and/or deletion during their development in the thymus (i.e., via

central tolerance mechanisms) are deleted or opposed in the periphery, thereby reducing the likelihood of autoimmune reactions. Peripheral tolerance is achieved through anergy and suppression mechanisms directed toward both T cells and APCs through the actions of regulatory T cells and the hormonal mediators they produce.

#### **4.2 Regulatory T Cells**

Two general subsets of regulatory T cells (Tregs) exist, i.e., ‘natural’ (nTregs) and ‘induced’ or ‘adaptive’ (iTregs). nTregs develop in the thymus and are fully functional once exported into the periphery. These cells represent 5-10% of the peripheral CD4<sup>+</sup> and 1% of the peripheral CD8<sup>+</sup> T cell populations and are identified experimentally by constitutive expression of the alpha chain of the IL-2 receptor (CD25<sup>+</sup>) and the transcription factor forkhead box protein 3 (FOXP3) [25-27]. FOXP3 expression begins early during thymic development, thereby initiating and maintaining a developmental program that specifies this Treg lineage during positive selection of high-affinity T-cell receptor ligands [27]. nTregs inhibit both proliferation and cytokine production by CD4<sup>+</sup> and CD8<sup>+</sup> T cells through a cell contact-dependent mechanism [28].

Inducible Tregs acquire their regulatory function in the periphery where they develop from naïve CD4<sup>+</sup> or CD8<sup>+</sup> T cell precursors following antigenic stimulation [26]. Thus, CD4<sup>+</sup> T regulatory type 1 cells (Tr1) and T helper type 3 regulatory cells (Th3) emerge to exert their suppressive effects in a cell contact-independent manner mainly via secretion of immunosuppressive cytokines [28]. Tr1 cells secrete interleukin(IL)-10, whereas Th3 cells secrete both IL-10 and transforming growth factor(TGF)-β [28].

Peripheral tolerance centered on the APC compartment is mediated through maturational arrest. Immature APCs under the influence of IL-10 and TGF- $\beta$  can render T cells anergic or unresponsive. Glucocorticoids (GC), TGF- $\beta$  and IL-10 down-regulate the expression of antigen presenting machinery, such as MHC class II and co-stimulatory proteins including CD54, CD80 and CD86 [29,30]. The resulting semi-mature APC fails to produce inflammatory cytokines but may produce IL-10 and TGF- $\beta$ , thereby influencing naïve T cells to differentiate into either inducible Tr1 or Th3 regulatory cells [31]. Thus the combined influence of the hormonal triad comprised of the GC, TGF- $\beta$  and IL-10, is to exert pressure toward sustaining immunological self-tolerance by actively suppressing inflammatory immune responses in the periphery.

## **5. Malnutrition and Immune Competence**

Both the innate and adaptive components of immune competence are depressed in acute PEM. Impairment of innate defences affects physical barriers, phagocytic cell numbers and activity, natural killer cell activity, the acute phase response and the complement system, as reviewed elsewhere [6]. Acute malnutrition consistently depresses the cell-mediated form of immune competence in children and in relevant rodent models whereas the impact on humoral immunity is less predictable [5,32-34]. Some of the mechanisms that contribute toward the cell-mediated immune depression in PEM include lymphoid involution, imbalances of T cell subsets and an overabundant presence of naïve T cells.

Lymphoid involution, a characteristic of acute malnutrition, involves both primary and secondary lymphoid organs which exhibit a degree of wasting that is

disproportionately large relative to whole body wasting [35]. In experimental rodent models of acute malnutrition, reduction in T and B lymphocyte numbers within secondary lymphoid organs exceeds the involution of the re-circulating (surveillance) lymphocyte pool [36]. Although lymphoid involution plays a role in malnutrition-associated immune depression, this phenomenon does not appear to be a determining factor. For example, triiodothyronine intervention studies revealed that loss of cell-mediated and humoral immune function can be prevented despite lymphoid atrophy and weight loss in rodent models of weanling malnutrition [37-39].

Within the involuted T cell population, both CD4<sup>+</sup> and CD8<sup>+</sup>, there is evidence of an overabundance of cells exhibiting a quiescent or naïve phenotype (i.e., CD45RA<sup>+</sup>) in the blood and secondary lymphoid organs of acutely malnourished weanling mice [40-42]. A similar phenomenon is apparent in the blood of acutely malnourished children [43]. Therefore, such an imbalance, in favour of quiescent or sluggish T cells, may limit T-dependent immune responses and play a role in sustaining the depressed adaptive immune competence that is associated with acute malnutrition [40].

The majority of research initiatives pertaining to malnutrition-associated immune depression have been T cell-centric. However, the evidence supporting a naïve shift within the T cell compartment provoked studies centered on the dendritic cell (DC) which possesses the unique ability to present antigen to naïve T cells, thereby initiating primary T-dependent immune responses [17,44]. It is postulated that DC numbers limit primary cell-mediated inflammatory immune competence in acute weanling malnutrition [44]. This proposition was based on evidence of stochastic interactions between T cells and inflammatory DC which are rate limiting to T-cell priming in vitro [45] and on

evidence that T cells compete for access to antigen-pulsed DC in vivo [46]. In this connection, splenic DC numbers decline to a greater extent than total nucleated splenocytes in the advanced stages of combined protein and energy deficiency in the weanling mouse [47]. However, interestingly, adoptively transferred syngeneic DC restored cell-mediated inflammatory immune competence in a weanling mouse model of incipient kwashiorkor, whereas adoptive transfer of syngeneic T cells failed to influence cell-mediated immune competence in the same experimental system [44]. Overall, there is a contribution by the T cell compartment to malnutrition-associated immune depression, but the influence of acute malnutrition on primary cell-mediated immunity appears to center fundamentally within the APC compartment [44]. Further, it remains possible that peripheral immune suppression contributes to depressed T-dependent immune competence and, in this connection, within the involuting splenic DC population a large proportion of cells express a tolerogenic surface phenotype [47]. This identifies a subset of DCs with a functional bias toward the promotion of peripheral tolerance and, interestingly, was apparent in the advanced stage of protein and energy deficit wherein losses of lean tissue would inevitably liberate a large amount of self-antigens [47].

## **6. Polarization of Immune Responses in PEM**

The classic observation of depressed inflammatory cell-mediated immunity and less predictably impacted humoral responses in acute PEM may be explained, in part, by the proposition of a type-2-biased form of immune competence in wasting pathology [6]. In this connection the bioactivity of the type-1 polarizing cytokine, IFN- $\gamma$ , is depressed in weanling mouse models of acute PEM [48]. Downstream of cytokine production, the

serum immunoglobulin profile also exhibits a type-2 biased polarization in acute PEM, a phenomenon which can be attributed to the influence of polarizing cytokines acting as regulators of immunoglobulin class switching [49]. IgE is the quintessential type-2 immunoglobulin in both humans and mice, whereas IgG1 also represents a type-2 subclass in the mouse [11,49]. The serum immunoglobulin profile exhibits high concentrations of IgE in acutely malnourished children and weanling mice [33,50] and high levels of IgG1 in acutely malnourished weanling mice [50]. Conversely, blood concentrations of the type-1 immunoglobulins, IgG2a and IgG3 [49], are unaffected by wasting pathology [50].

## **7. Glucocorticoids and Immune Function**

GC hormones, cortisol in humans and corticosterone in rodents, are synthesized in the adrenal cortex and represent the final hormone produced following stimulation of the well-defined hypothalamic-pituitary-adrenal axis [51]. Some of the physiological roles of GC hormones include resistance to stress, regulation of intermediary metabolism and complex interactions with the immune system which are mainly anti-inflammatory and immunosuppressive [52]. GCs exert these effects by diffusing freely through the cell membrane and binding in the cytoplasm to the glucocorticoid receptor (GR), a ligand-dependent transcription factor [52,53]. In the absence of bound hormone, the GR forms a complex with heat shock proteins and immunophilins in the cytoplasm, which act as chaperones [54,55]. However, once GCs are bound to the GR, the transcription factor dissociates from the heat shock protein complex, translocates to the nucleus and binds to glucocorticoid response elements (GRE) present in the promoter and enhancer regions of

a variety of genes [56,57]. Some GC-responsive genes are directly upregulated through GR/GRE binding, but the GR can also regulate gene expression by interfering with other transcription factors such as NF- $\kappa$ B, AP-1, NF-AT, CREB and Stat5 [53,55]. The majority of the immunosuppressive and anti-inflammatory effects of the GC are mediated through the inhibition NF- $\kappa$ B and AP-1, which activate inflammatory genes [52,55,58]. Thus a large number and diversity of genes is impacted by the GC. Immunologically, the outcome is inhibition of synthesis and secretion by T lymphocytes and APCs of a multitude of cytokines, primarily of an inflammatory nature, including IL-1, IL-2, IL-3, IL-5, IL-6, IL-8, IL-12, TNF- $\alpha$ , IFN- $\gamma$  and GM-CSF by T lymphocytes and APCs [57,59-63]. Additionally, GCs appear to oppose the actions of inflammatory cytokines by increasing the protein levels of their natural inhibitors, such as the IL-1 receptor antagonist (IL-1ra) [64] and the soluble IL-1 type II receptor [65].

GCs induce arrest in the maturation of APCs. Thus, GCs enhance the immature APC phenotype which includes low levels of surface MHC class I and II molecules and co-stimulatory molecules such as CD40, CD80 and CD86 in addition to maintaining a high antigen uptake capability [63,66-68]. Consequently, following exposure to GCs, APCs exhibit a reduced ability to present antigens and decreased expression of co-stimulatory molecules relevant for T cell activation [66,67].

GCs exert strong direct and indirect influences on the balance between type-1 type-2 CD4<sup>+</sup> effector T cell responses. Production of the type-2 polarizing cytokine, IL-4, is enhanced by GC [69-72], whereas IFN- $\gamma$  production is directly suppressed, thereby providing a direct and indirect mechanisms through which GC suppress the development of inflammatory cell-mediated immune responses [60-62,73]. Further, GCs indirectly

decrease the capacity of T cells to produce the inflammatory cytokine, IFN- $\gamma$ , by inhibiting the production of IL-12, an APC-derived type-1 promoting cytokine [51,63,69,70,74]. In this connection, GCs also down-regulate the expression of the IL-12 receptor on T and NK cells, thereby rendering these cells less receptive to IL-12 signaling [51]. Thus, following exposure to GC, a progressive shift takes place from a cellular Th1-type to a humoral Th2-type immune response [63].

GCs contribute to immune tolerance through direct mechanisms including the triggering of T cell apoptosis or by mediating T cell anergy. Indirect tolerance mechanisms involve the maturational arrest of APCs [63], which can further promote T cell anergy or the development of inducible regulatory T cells [75]. Further, GCs enhance the expression of IL-10 (by macrophages, immature DC and CD4<sup>+</sup> T cells), a tolerogenic cytokine that induces regulatory T cells [63,75]. Therefore, GCs participate in the differentiation and expansion of IL-10-producing regulatory T cell populations through their combined inhibitory actions on APC maturation and enhancement of APC- and T cell-mediated IL-10 secretion [63]. Further, GCs up-regulate the expression of FOXP3, a key transcriptional repressor that is uniquely expressed by regulatory T cells [76,77]. Therefore, in addition to their potently anti-inflammatory characteristic, these hormones also contribute to immunological tolerance.

## **8. Transforming Growth Factor- $\beta$ and Immune Function**

TGF- $\beta$  is a broadly anti-inflammatory and immunosuppressive cytokine that exists in three mammalian isoforms namely TGF- $\beta$ 1, - $\beta$ 2 and - $\beta$ 3 [78]. All tissues produce at least one TGF- $\beta$  isoform, and a variety of different cell types express

receptors for this cytokine [78,79]. Therefore, virtually every cell has the potential of both producing and responding to this cytokine, affording TGF- $\beta$  with a unique potential to influence every physiological system. Hence, its activation and subsequent actions are tightly regulated [80]. TGF- $\beta$  activation is not regulated by controlling its production or receptor expression but, instead, is regulated by mechanisms that convert latent TGF- $\beta$  into its active form [80]. Initially, TGF- $\beta$  is produced in a form consisting of an active protein non-covalently bound to latency associated protein (LAP, derived from the N-terminal of the TGF- $\beta$  precursor) which remains in the cell [78,80]. This complex is referred to as the small latent complex (SLC) and, in order for TGF- $\beta$  to become activated, it must be cleaved from LAP. The SLC is secreted from cells following the binding of a third protein referred to as latent TGF- $\beta$ -binding protein (LTBP), thus forming the large latent complex (LLC) that prevents TGF- $\beta$  from interacting with its receptor [78,80,81]. Several isoforms of LTBP exist and it is proposed that these proteins serve as structural components of the extracellular matrix (ECM) and modulators of TGF- $\beta$  availability [78]. Via binding sites on the LTBP, the LLC is bound to collagen and other tissue matrix proteins, thereby concentrating TGF- $\beta$  to elastin fibrils and fibronectin-rich pericellular fibers [80,82]. In this connection, the ECM may serve as a storage deposit for TGF- $\beta$ , from which it is readily accessible for activation. Proteases such as plasmin and matrix metalloproteinases -2 and -9 release the LLC from the tissue matrix by targeting the hinge region in the LTBP, thereby liberating the remainder of the LLC which is now free to bind to surface receptors and to release TGF- $\beta$  [78,80]. Once activated, all three TGF- $\beta$  isoforms use the same receptor, which presents in three forms, i.e. RI, RII and RIII [78,80]. As there is no known intracellular signaling pathway

associated with RIII, both the membrane bound and soluble forms of this receptor are regarded as inhibitors of TGF- $\beta$  [80]. TGF- $\beta$  exerts its biological effects as a homodimer that binds to a heterodimeric complex comprised of TGF $\beta$ RI and TGF $\beta$ RII. Once the cytokine is bound, intracellular kinase domains of the receptor are activated and phosphorylate SMAD (mothers against decapentaplegic homologue) proteins which ultimately transduce the signal to the nucleus [83].

TGF- $\beta$  is implicated in multiple biological processes including angiogenesis, wound healing, apoptosis and fibrosis [78]. Immunologically, TGF- $\beta$  exerts an inhibitory effect on cells of both the innate and adaptive arms of the immune system including T lymphocytes, NK cells, APCs, polymorphonuclear leukocytes and mast cells [78,84]. Broadly, the impact of TGF- $\beta$  on immune function is suppressive and anti-inflammatory [84,85], but the impact of this cytokine on inflammatory status is highly influenced by the cytokine microenvironment [85,86]. Stimulation of naïve T cells in the presence of TGF- $\beta$ 1 leads to the induction of FOXP3 expression, thereby generating inducible Tregs [83,87,88]. However the addition of IL-6 in conjunction with TGF- $\beta$  upregulates the expression of the transcription factor retinoic-acid-receptor-related orphan receptor- $\gamma$ t, a key regulator necessary for the differentiation of naïve CD4<sup>+</sup> T cells to become T<sub>H</sub>17 cells which are associated with chronic inflammation and autoimmune diseases [83,84,86,87]. In light of the dual role that TGF- $\beta$  plays in driving Treg and T<sub>H</sub>17 differentiation, this cytokine has been regarded as a key player in regulating both classic inflammatory defence responses and autoimmune diseases.

TGF- $\beta$  exerts an inhibitory effect on APCs, thereby providing an indirect means of down-regulating T cell activation and differentiation. TGF- $\beta$  inhibits the activation of

macrophages and their subsequent production of inflammatory mediators [89,90].

Further, APC maturation from a highly endocytic non-antigen presenting cell type to that of a low-uptake and high antigen presenting type is down-regulated through the actions of TGF- $\beta$  [83,90]. Therefore the immature APC, with a phenotype of high intracellular levels and low surface expression of MHC class II and co-stimulatory molecules (CD80 and CD86) persists under the influence of TGF- $\beta$  [90,91], an outcome similar to the effects of GCs [63,66-68,72].

With respect to T cells, TGF- $\beta$  exerts an anti-proliferative effect which is largely attributed to the inhibition of IL-2 production by T lymphocytes [90]. TGF- $\beta$  also acts to impair T cell (CD4<sup>+</sup> and CD8<sup>+</sup>) differentiation and proliferation and most, if not all, effector T cell functions [83,90]. Thus, CD8<sup>+</sup> T cells fail to acquire effector CTL status (i.e., fail to express perforin and granzyme) and CD4<sup>+</sup> T cells fail to differentiate to Th1 or Th2 effector status [83,90,92]. The influence of TGF- $\beta$  on Th1 cell development is further impacted by the downregulation of IL-12 receptor  $\beta$  chain expression and subsequent responsiveness to this cytokine [93]. Additionally, the expression of the key transcriptional regulators for Th1 and Th2 development, T-bet and Gata-3, respectively, are inhibited through the actions of TGF- $\beta$  [83,94]. Further, the expression of the polarizing Th1 cytokine, IFN- $\gamma$ , and the APC cytokine that induces its production, IL-12, are both suppressed by TGF- $\beta$  [95,96].

## 9. Interleukin-10 and Immune Function

Interleukin(IL)-10 is an immunosuppressive cytokine with broad anti-inflammatory properties. This dominant suppressive function is illustrated by the phenotype of IL-10 deficient mice which develop colitis in the presence of normal gut flora [97,98]. The role of IL-10 appears to be the containment and suppression of inflammatory immune responses to pathogens so as to down-regulate adaptive inflammatory responses otherwise typified by overproduction of IFN- $\gamma$  and TNF- $\alpha$ , thereby preventing tissue damage to the host [99,100]. Further, this cytokine is able to influence the actions of cells participating in both the innate and adaptive immune responses, thus, limiting inflammatory cascades and restoring homeostasis via negative feedback mechanisms [101-103]. Therefore IL-10 plays an important role in controlling the initiation and progression of inflammatory defence responses and autoimmune diseases.

Several cell types of both an immunological and non-immunological nature are capable of producing IL-10 including eosinophils, keratinocytes, adipocytes, mast cells, macrophages, DC, B cells and various T cell subsets (i.e., CD8<sup>+</sup>, CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>, Tr1, CD4<sup>+</sup> Th1 and Th2 cells) [99,104-107]. The IL-10 receptor is comprised of two subunits. The ligand-binding subunit, IL-10R $\alpha$ , or IL-10R1, is constitutively expressed by most hemopoietic cells (a few hundred per cell) and can also be induced on the surface of many non-hemopoietic cells [101]. The accessory subunit, IL-10R2, or IL-10 $\beta$ , is constitutively expressed in most cells and tissues [108,109] and plays an important role in signal transduction by recruiting Jak kinase into the signaling complex [101]. As indicated, signal transduction through the IL-10 receptor is mediated by the Janus kinase/STAT pathway, whereby Janus kinase phosphorylates and activates the latent

transcription factors, STAT1 and STAT3, with subsequent formation of three different DNA binding complexes [107]. These binding complexes attach to specific sites on the nuclear DNA, thereby leading to the sequential transcription of specific genes [107].

Additionally the ability of the transcription factors NF $\kappa$ B and AP-1 to stimulate the transcription of inflammatory genes is reduced by IL-10, thus providing a potent anti-inflammatory influence [101,110]. NF $\kappa$ B activation is inhibited by IL-10 through at least two separate mechanisms, i.e. by inhibition of I $\kappa$ B kinase activation and by directly inhibiting NF $\kappa$ B DNA binding [101]. It is largely through the inhibition of NF $\kappa$ B activation that IL-10 exerts its anti-inflammatory effects. Ultimately the cellular microenvironment dictates whether the immunosuppressive or immunostimulatory effects of IL-10 will prevail, but in most situations, the immunosuppressive properties dominate [111].

In addition to eliciting an anti-inflammatory effect by reducing the production of inflammatory mediators such as nitric oxide, free radicals and prostaglandins [101,103], a large body of evidence indicates that IL-10 exerts a strong regulatory effect on Th1 lymphocytes and APCs. The influence of this cytokine on APCs, specifically macrophages and DC, is to inhibit expression, production and secretion of inflammatory cytokines including TNF- $\alpha$ , IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, IL-12 and IL-18 [100,101, 103,104,112]. Conversely, IL-10 enhances the expression of the natural antagonists of two inflammatory cytokines, IL-1 and TNF- $\alpha$ , via the IL-1ra and soluble p55 and p75 TNF receptors, respectively [101,113]. Further, IL-10 attenuates the surface expression of TNF receptors and promotes their shedding into the systemic circulation, thereby blocking the inflammatory actions of TNF- $\alpha$  [103]. Production of most inducible

inflammatory chemokines that recruit monocytes, DC, neutrophils and T cells, i.e. MDC, MCP1, MCP5, Rantes, IP-10 and MIP-2, is suppressed by IL-10 [100,101,103,104]. Additionally, autocrine IL-10 signaling in DC can inhibit their chemokine production and prevent their trafficking to lymph nodes with resultant inability to recruit and induce the differentiation of Th1 cells from naïve precursors [114].

The general immunosuppressive character of IL-10 is demonstrated through the influence on tolerogenic APCs and via the inhibition of Th1 effector T cell responses [103]. A large proportion of the anti-inflammatory influence exerted by IL-10 is via its suppressive influence on IFN- $\gamma$  which is mediated in part through suppression of IL-12 production by mononuclear phagocytes, thereby down-regulating the ability of these cells to activate the Th1 subset [115,116]. Independently of the suppression of APC-derived cytokines, IL-10 has been shown to inhibit IL-2 and IFN- $\gamma$  production by T cells [103]. In this connection, IL-10 plays an important role in cross-regulation of the Th1 and Th2 response. In conjunction with IL-4, IL-10 suppresses the expansion of the Th1 cell population [11]. Therefore, IL-10 can act as a polarizing Th2 cytokine and a physiological regulator of the Th1 response.

IL-10 down-regulates expression of MHC class II proteins, co-stimulatory molecules (i.e. CD54, CD80 and CD86) and adhesion molecules on the APC surface, thereby inhibiting effective antigen presentation [100,101,103]. The outcome of this influence is a negative impact on proliferative responses and cytokine production by T cells [103,111]. IL-10-mediated inhibition of maturation is reported to render APC tolerogenic [117]. The impairment of APC maturation and subsequent tolerogenic cellular

characteristics induced by IL-10 is an influence that is in common with the effects of both the GCs [63,66-68,72] and TGF- $\beta$  [90,91].

## **10. Hormonal Response to PEM**

Structurally, hormones fall into one of three categories: polypeptide, amide or steroid and cytokines, which are exclusively polypeptide-based molecules, constitute a component of this hormone category and may be regarded as such [118]. Hormones function in an autocrine, paracrine and/or endocrine manner and, thus, the same chemical substance could be classified differently depending upon the physiological setting and mechanism of action under consideration [119]. In this connection, cytokines can act in an endocrine as well as paracrine-autocrine fashion, whereas classical endocrine hormones can also exhibit paracrine-autocrine activities [118,119]. In response to acute PEM, endocrine hormones mediate a reorganization of the systemic distribution of energy and substrates towards conservation while promoting glucose homeostasis and the metabolism of fat [6]. Endocrine hormones act to exert systemic control over metabolism, whereas the network of autocrine and paracrine hormones, i.e., the cytokines, provide fine control over physiological functions that, collectively, constitute and impact immune defence [6]. Deficits of protein and/or energy elicit an adaptive response whereby blood concentrations of leptin, thyroid hormones (triiodothyronine and thyroxine), insulin and IFN- $\gamma$  are decreased whereas GC, TGF- $\beta$  and IL-10 concentrations are elevated [6,39,120,121]. The hormonal response to acute malnutrition has the potential to profoundly impact immune function, and the triad of anti-inflammatory and tolerogenic hormones that dominate the advanced stages of acute pre-pubescent malnutrition, namely

GC, TGF- $\beta$  and IL-10, are of particular interest. These hormones share a common permissive influence on type-2 polarization of T cell responses via inhibition of IFN- $\gamma$  [61,62,73,95,96,103] and through a direct suppressive effect on APC maturation [63,68,90,91,103], and create a cytokine microenvironment that would be expected to support the development of regulatory T cells.

The best evidence implicating endocrine influences on malnutrition-associated immune competence derives from experimental hormonal interventions that have successfully either prevented or reversed immune depression despite continued weight loss [37,59,122]. For example, exogenous leptin administered to mice sensitized during a 48-hour starvation period prevented the development of depressed primary cell-mediated immune competence [122]. This is attributed to an impact of leptin on T cell cytokine secretion, mainly increasing IFN- $\gamma$  (Th1) while depressing IL-4 (Th2) production [122]. Additionally, triiodothyronine supplementation of weanling mice subjected to experimental acute PEM was found to normalize the blood levels of triiodothyronine and to improve acquired humoral and cell-mediated responses [37]. Further both adrenalectomy and GC receptor blockade reversed the impaired macrophage function that is characteristic of protein-deficient mice [59].

Increased hormonal blood concentrations in advanced-stage acute PEM provide evidence of regulation rather than loss of control, and in this connection, the influence of the elevated hormonal triad comprised of GC, TGF- $\beta$  and IL-10 is of interest in wasting pathology. Considering the potential immunological implications of altered hormonal concentrations in response to acute weanling PEM, it is of interest to pursue an understanding of the hormonal underpinning that may contribute toward the initiation,

and/or the ongoing support, of malnutrition-associated depression in cell-mediated immune competence.

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## Chapter Two

### Introduction and Objectives

Immune depression in acute (i.e., wasting) PEM is recognized as the most common cause of immune suppression worldwide and is prevalent in both developing and industrialized countries [1]. Inflammatory cell-mediated immune competence is consistently depressed whereas humoral competence is less predictably affected in the malnourished [2,3]. Thus, the impact of acute PEM on aspects of adaptive immune competence may reflect an underlying impact on the polarization of adaptive immune responses. Type one polarized responses defend the intracellular spaces via IFN- $\gamma$  production and thus support cell-mediated immunity [4,5]. Type two polarized responses defend extracellular spaces via IL-4 production and thus support humoral immunity [4,5]. In this connection, blood mononuclear cells and T cells isolated from acutely malnourished children suffering various gastrointestinal and respiratory infections exhibited reduced expression of IFN- $\gamma$  mRNA coupled with an elevation in IL-4 mRNA expression and no effect on T cell IL-4 expression [6,7]. Further, blood levels of IFN- $\gamma$  are depressed [8] in murine models that reproduce the critical features of acute pre-pubescent malnutrition, which consistently depresses inflammatory cell-mediated immune competence [9-11]. Further evidence supporting malnutrition-associated type two polarization stems from the serum immunoglobulin profile in acute PEM, wherein the concentration of IgE class antibody, the quintessential type 2 immunoglobulin, is elevated in malnourished children [12] and weanling mice [13]. Despite the aforementioned evidence, a definitive study wherein the influence of acute malnutrition

on the balance between type 1 and type 2 polarization has not been conducted. This has important implications for an understanding of the depression in inflammatory capacity associated with acute pre-pubescent PEM.

**Objective 1: To determine the influence of acute pre-pubescent malnutrition on the in vitro and in vivo production of the type 1 and type 2 polarizing cytokines, IFN- $\gamma$  and IL-4, respectively.**

Further to the well-documented depression in inflammatory immune competence [2,3] the impact of wasting malnutrition on the anti-inflammatory form of immune competence is virtually unknown. Acutely malnourished children [14] and rodents [15,16] exhibit a profound increase in the blood concentrations of the anti-inflammatory and immune suppressive glucocorticoid hormones at least in the advanced stages of weight loss. Additionally, the anti-inflammatory and immune suppressive cytokines, TGF- $\beta$  and IL-10, also exhibit elevated blood levels in mouse models of the advanced stage of acute pre-pubescent malnutrition [17]. The glucocorticoids, IL-10 and TGF- $\beta$  comprise a network of partially redundant anti-inflammatory hormonal mediators effective against diverse innate and adaptive immune defence elements and are widely regarded as the backbone of peripheral self-tolerance [18-22]. Thus, these three hormones may work in concert, at least during the advanced stages of weight loss, directing the depressed inflammatory cell-mediated form of immune competence that is characteristic of PEM. However, little is known about the early stages of weight loss in response to nutritional deficits of protein and/or energy or the time kinetics of the rise in blood levels of these hormones. If blood levels of anti-inflammatory and immune

suppressible hormones were found to rise sufficiently early in response to acute deficits of protein and/or energy, this would have implications as to their possible role in the initiation of inflammatory immune depression.

**Objectives Two - Four: To determine when the rise in blood corticosterone (objective two), TGF- $\beta$  (objective three) and IL-10 (objective four) occurs in acute weanling PEM and to confirm the previous reports of elevated levels in the advanced stages of weight loss. As an additional component of objective three, the systemic rate of constitutive IL-10 production will be assessed in both the early and advanced stages of weight loss.**

IL-10 acts as a key regulator of both the innate and adaptive arms of the immune system [23,24]. Thus the caveat to objective four, i.e., constitutive IL-10 production, pertains to a primary tier of anti-inflammatory immune competence. Subsequent production of this cytokine in response to stimuli may be achieved through contributions by the innate and adaptive arms of the immune system, thus representing a redundant secondary tier of anti-inflammatory and tolerogenic immune regulation. Consequently, the following additional objectives are addressed herein:

**Objectives Five and Six: To determine the influence of acute PEM on second-tier innate-type (objective five) and adaptive-type (objective six) IL-10 production in response to stimuli that elicit IL-10 production beyond constitutive cytokine production levels.**

Having determined the primary and secondary tiers of IL-10 production in acute pre-pubescent malnutrition, the cellular source(s) of this cytokine remain elusive and their identification is complicated by the anatomical dispersion and diverse cell types capable of producing this cytokine [23, 25-27]. Investigation of the cellular sources of IL-10 in acute malnutrition is of interest in view of the high constitutive levels of this cytokine that are reported in the blood in the advanced stages of pre-pubescent wasting disease [17]. Thus, utilizing the strategy of targeting the anatomical sites exhibiting a high expression of IL-10 mRNA, will provide insight into the potential cellular sources responsible for the aforementioned phenomenon in acute malnutrition.

**Objective Seven: To determine the impact of acute pre-pubescent PEM on IL-10 gene expression in lymphoid tissues wherein IL-10 producing cells congregate, namely the spleen, bone marrow and small intestine.**

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### Chapter 3

#### **Effector/memory T cells of the weanling mouse exhibit Type-2 cytokine polarization in vitro and in vivo in the advanced stages of acute energy deficit.**

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## **Abstract**

The objective was to determine whether the polarizing cytokine profile of the effector/memory T cell compartment reflects the profound decline of cell-mediated inflammatory competence that characterizes acute pre-pubescent malnutrition. Weanling C57BL6/J mice were permitted free access to a complete purified diet, free access to an isocaloric low-protein diet or restricted intake of the complete diet for 14 days. First, interleukin(IL)-4 and interferon(IFN)- $\gamma$  concentrations generated in vitro by splenic and nodal effector/memory T cells were assessed following exposure to plate-bound anti-CD3. Second, net systemic production of IFN- $\gamma$  and IL-4 by the effector/memory T cell compartment was assessed by the in vivo cytokine capture assay following anti-CD3 stimulation. In vitro stimulation generated less IFN- $\gamma$ , but more IL-4, by T cells from the restricted-intake group relative to age-matched controls (P=0.002 and 0.05). Similarly, in vivo stimulation generated low serum levels of antibody-captured IFN- $\gamma$  in the restricted-intake group vis-à-vis age-matched controls (P=0.01), while the IL-4 response was sustained (P=0.39). By contrast, the 14-day low-protein model exhibited no change in T cell cytokine signature either in vitro or in vivo. However, following extended consumption of the low-protein diet (26 days), carcass energy losses exceeded those of the 14-day protocol and serum levels of in vivo antibody-captured IFN- $\gamma$  were low after anti-CD3 challenge relative to age-matched controls (P=0.02), while levels of captured IL-4 remained unaffected (P=0.07). Acute weanling malnutrition elicits a Type 2 polarizing cytokine character on the part of the effector/memory T cell compartment, but only in the most advanced stages of energy decrement.

## **1. Introduction**

Acute (i.e., wasting) pre-pubescent deficits of protein and/or energy in their most severe forms, i.e. marasmus and kwashiorkor, are associated with up to two million largely infection-related deaths annually among children under the age of five years, and this toll is undoubtedly exceeded by the additional burden of infection-related morbidity [1]. Depressed immune competence is widely accepted as the link between malnutrition and susceptibility to opportunistic infection [2] and repairing inflammatory immune competence has been identified authoritatively as one of three preferred complementary approaches to reducing the burden of malnutrition-associated infection [3]. Rational, targeted immunological interventions, however, must await substantive improvement in mechanistic understanding of the cellular basis underlying malnutrition-associated immune depression [2].

It is firmly established that acute pre-pubescent malnutrition produces a profound depression in adaptive inflammatory cell-mediated immune competence, whereas humoral immune competence is less predictably affected [2]. This phenomenon may find some basis in the capacity to produce cytokines within the categories designated as Type 1 and Type 2 [4,5] that regulate adaptive immune competence. Broadly, Type 1 cytokines promote cell-mediated immune responses and the production of opsonizing and complement-fixing subclasses of IgG antibody that support this type of response [6] which is designed for defence against intracellular pathogens [5,7]. By contrast, type 2 cytokines promote production of antibodies whose main function is to provide protection in the extracellular space [5,7].

Interferon (IFN)- $\gamma$  is a signature type 1 cytokine and serves to polarize acquired immune responses in a cell-mediated direction [5,7]. Type 2 cytokines include interleukin (IL)-4, -5, -6, -10 and -13, and IL-4 is regarded as the primary polarizing cytokine of this group [5,7]. Both IFN- $\gamma$  and IL-4 arise from a variety of cellular sources including T cells [4,7]. To acquire the capacity to produce cytokines such as IFN- $\gamma$  and IL-4 that reflect the end-stage polarity of an adaptive immune response, T cells must achieve effector/memory status [8]. Naïve T cells require 24-48 hours of stimulation to commit to effector polarization in vitro [8,9] and can commit to effector differentiation only if subjected to simultaneous stimuli through the T cell receptor and through CD28 [10]. By contrast, effector/memory T cells respond with cytokine production after only a few hours of stimulation in vitro [9] and stimulation through the T cell receptor, alone, is sufficient to elicit a response [10]. Effector/memory T cells exhibit mutually exclusive Type 1 and Type 2 polarities reflective of the microenvironment within which their activation beyond naivety took place [7]. The cytokine profile of the effector/memory T cell compartment, therefore, provides a physiological index of the systemic balance between type 1 and Type 2 immunological polarities. In turn, this index provides a tool with which to probe the proposition [11,12] that a form of immune competence regulated toward Type 2 cytokine polarity underlies the particular sensitivity of cell-mediated immunity to acute malnutrition.

Depressed IFN- $\gamma$  production in response to polyclonal T cell mitogen stimulation is reported on the part of splenic mononuclear cells from acutely malnourished adolescent rats [13]. The same finding is reported in a study of spleen cells from young adult mice under conditions, in vitro, in which IL-4 production was unaffected [14]. Although clear-

cut, the cited investigations were not designed to identify the influence of acute malnutrition pathologies specifically on the cytokine polarity of the effector/memory T cell compartment. This is substantially because in vitro response times exceeding 24 hours, such as those used in the cited studies, permit activation of naïve T cells [9]. Moreover, the cited studies are not directly relevant to the pre-pubescent stage of life. Therefore, the first objective of this investigation was to determine the influence of metabolically distinct forms of acute pre-pubescent malnutrition, mimicking marasmus and incipient kwashiorkor, on the polarity of the effector/memory T cell compartment as indicated by the production of IFN- $\gamma$  and IL-4 in short-term culture.

It is desirable to determine whether results derived in vitro reflect the reality of the intact organism. In this connection, one study of acutely protein-deficient young adult mice records depressed production of IFN- $\gamma$  mRNA in vivo in response to experimental tuberculosis [15]. However, the design of the latter investigation did not permit identification of the cellular source of the cytokine. Moreover, no report pertaining to the influence of acute malnutrition on the balance between Type 1 and Type 2 cytokines, regardless of cellular source, appears available based on assessments of cytokine production in vivo.

The in vivo cytokine capture assay was originally designated the Cincinnati cytokine capture assay and is, in effect, a competitive binding procedure permitting assessment of net rate of cytokine production on a systemic level [16,17]. No other technique is available for this purpose. The method centers on a monoclonal biotin-conjugated anti-cytokine antibody of IgG class that is used to trap a small fraction of cytokine within the blood compartment. The accumulation of the resulting immune

complex during a period of a few hours is subsequently quantified. The assay has been validated in the mouse for several cytokines including IFN- $\gamma$  and IL-2, -4, -6 and -10 [17], and permits simultaneous assessment of more than one cytokine as has been demonstrated for IFN- $\gamma$  and IL-4 in the mouse [16]. To date, the assay has provided valuable insights into the immunobiology of cytokines including their roles in infectious and other inflammatory conditions [17]. In relation to the purpose of the present investigation, systemic cytokine production specifically by the effector/memory T cell compartment is readily quantified by means of stimulation with anti-CD3 [16,17]. The second objective, and ultimately the main goal, of this investigation was to use the in vivo cytokine capture assay to assess the influence of diverse forms of acute weanling malnutrition on the net systemic production of IFN- $\gamma$  and IL-4 by the effector/memory T cell compartment in response to polyclonal stimulation imposed in vivo.

## **2. Materials and methods**

### *2.1. Animals and facilities*

Male and female C57BL/6J mice were used from an in-house breeding colony derived from animals of the Jackson Laboratory (Bar Harbor, ME). Caging and housing conditions were exactly as described previously [18-23], and the investigation was approved by the Animal Care Committee of the University of Guelph in accordance with the Canadian Council on Animal Care.

### *2.2. Diets, feeding protocols and experimental design*

Mice were weaned at 18 days of age and acclimated for 1 day with free access to the complete purified diet described elsewhere [24]. A typical proximate analysis for this diet is 92.3% dry matter, 18.8% crude protein, 8.1% ether extract, 2.6% ash, 3.1% crude fibre and 17.0 kJ/g gross energy [25]. At 19 days of age, each mouse was randomly allocated to one of four experimental groups, namely an age-matched control group that consumed the complete diet ad libitum, a group that consumed the complete diet in restricted daily quantities, a group given free access to an isocaloric low-protein purified diet and a zero-time control group that was examined at 19 days of age to permit discrimination between diet- and ontogeny-related phenomena. The quantity of diet given the mice in the restricted intake group was calculated daily as described previously by this laboratory [26] with a view to achieving a daily loss of 1.5-2% of initial body weight throughout the 14-day experimental period. As described elsewhere [23,25], the purified low-protein diet contained 0.6% crude protein (as fed) and was prepared by replacement of most of the egg white (80% crude protein; U.S. Biochemical, Cleveland, OH) of the complete diet with an equal weight of cornstarch (ICN Biomedicals, Inc., Aurora, OH). During the acclimation and experimental periods, animals were housed individually. In addition, all animals had free access to clean tap water, and coprophagy was permitted.

Two experiments were performed in which the three groups other than the zero-time control were maintained on their respective regimens for 14 days, i.e. from 19 through 33 days of age. Animals included in the first experiment provided cells from the spleen and the mesenteric and inguinal lymph nodes for study in vitro, whereas the second experiment centered on cytokine production in vivo and the outcome was

assessed by means of a blood sample taken at the end of the 14-day experimental period. In the first experiment, a sample size of ten was achieved for each of the four groups except the restricted intake group for which  $n=8$ , and comparable numbers of males and females were included in each of the experimental groups. In order to obtain sufficient numbers of cells, pooled samples were required for mice subjected to the low-protein and restricted intake groups. Each pooled sample (2-4 mice for the low-protein group and 2-8 mice for the restricted intake group) included comparable numbers of males and females and constituted a single degree of freedom for the purpose of statistical analysis. The second experiment was conducted according to a 2 X 2 design in which diet and in vivo polyclonal T cell stimulation (using anti-mouse CD3) were the main effects. Eight mice (four males and four females) were included within each of the resulting eight groups of animals, and pooling was unnecessary. At the end of the 14-day experimental period, or at 19 days of age (zero-time control group), tissue was taken from each mouse, [i.e. mononuclear cells from secondary lymphoid organs (Experiment 1) or blood (Experiment 2)] and the carcasses were stored at  $-20^{\circ}\text{C}$  to await analysis.

A small supplementary experiment was performed in which 19-d-old C57BL/6J mice were given free access to either the complete diet or the low-protein diet for 26 days. Six mice, three males and three females, were assigned to each dietary group and the experiment centered on IFN- $\gamma$  and IL-4 production in vivo in response to stimulation with anti-CD3. The outcome of the experiment, therefore, was assessed by means of a blood sample taken at the end of the 26-day experimental period. Animal carcasses were stored at  $-20^{\circ}\text{C}$  to await analysis.

### *2.3. Procedure to obtain mononuclear cell suspensions combined from spleen and lymph nodes*

After measurement of body weight, mice were anesthetized with CO<sub>2</sub> and killed by cervical dislocation. The spleen as well as mesenteric and inguinal lymph nodes were removed aseptically, diced together and forced through a sterile stainless steel wire screen (100-mesh) into RPMI 1640 medium (Flow Laboratories, Mississauga, Canada) containing 10% heat-inactivated fetal bovine serum (Sigma Chemical, St. Louis, MO), 1mmol/L HEPES (ICN Biomedicals, Aurora, OH), 10<sup>5</sup> U/L penicillin and 100 mg/L streptomycin. A single-cell suspension was produced by discontinuous gradient centrifugation as described previously [23]. Cell numbers were determined using a hemocytometer, and viability assessed by eosin Y exclusion always exceeded 95%.

### *2.4. Stimulation of T cells to elicit cytokine production in vitro*

Falcon plates #3072 (Becton Dickinson Labware, New Jersey, US) were coated overnight at 4°C with 200 µL 0.01 M phosphate-buffered saline (PBS, pH 7.3) containing 5 µg/mL anti-CD3 (clone: 145-2C11; Cedarlane Laboratories, Hornby, Canada). After coating, plates were washed twice with PBS and each well received 2 x 10<sup>5</sup> viable mononuclear cells in 190 µL complete medium. Subsequently, 10 µL of PBS was added to half of the culture wells while the remaining test cultures received 10 µL of PBS containing anti-CD28 to achieve a final concentration of 20 µg/mL (clone: 37.51.1; Cedarlane Laboratories, Hornby, Canada). Negative control cultures were produced using wells not coated with anti-CD3 and the cells were cultured in fluids comprising 190 µL complete medium together with 10 µL PBS. All cultures were incubated at 37°C for 24 h.

After incubation, plates were centrifuged for 1 min at 200 x g, and supernatants from wells containing cultures of the same treatment group and stimulus were pooled and subdivided into aliquots. This permitted cytokine analyses without repeated freezing and thawing of samples which were stored at -80°C.

#### *2.5. Assay of cytokine concentrations generated in vitro*

Sandwich ELISA kits for assay of IL-4 and IFN- $\gamma$  (BD Biosciences, Mississauga, ON) were applied to samples as described by the manufacturer. Outcomes were quantified by optical density using a Vmax kinetic plate reader (Molecular Devices Corp., Menlo Park, CA) set for absorbance at 450 nm with wavelength correction based on absorbance at 570 nm. Only the linear portions of standard curves were used, and the reliability (intra-assay coefficient of variation) and detection limit of each assay were estimated as described elsewhere [27].

#### *2.6. Assessment of percentage CD3<sup>+</sup> cells in mononuclear cell suspensions from spleen and lymph nodes*

Analyses were performed using a Becton-Dickinson FACSCalibur flow cytometer equipped with BD CellQuest TM software (2001). Generic aspects of staining procedures in this laboratory, including Fc receptor blockade, are described elsewhere [21,23]. Viability before staining was determined by eosin Y exclusion and always exceeded 95%. Each analysis was based on at least 10<sup>4</sup> events after dead cells and residual erythrocytes were eliminated by gating on the basis of forward-angle light scatter.

Mononuclear cells were subjected to single-color analysis by means of phycoerythrin-conjugated anti-mouse CD3 $\epsilon$  (145-2C11, hamster IgG, eBiosciences, San Diego, CA) at a concentration of 0.2  $\mu$ g per 250 x 10<sup>3</sup> viable cells. Negative control samples were stained with biotin-conjugated hamster IgG (Cedarlane Laboratories, Hornby, ON) followed by phycoerythrin-conjugated streptavidin (Cedarlane Laboratories, Hornby, ON), respectively at concentrations of 0.2  $\mu$ g and 0.15  $\mu$ g per 250 x 10<sup>3</sup> viable cells. Cells were incubated with staining reagents in the dark on ice for 40 min, and were fixed in paraformaldehyde (20g/L), after which samples were analyzed within 7 days.

#### *2.7. In vivo cytokine capture assays for IL-4 and IFN- $\gamma$*

The assays were conducted basically as described [16,17]. Briefly, each mouse received, by intraperitoneal injection, 10  $\mu$ g of biotin-conjugated anti-mouse IL-4 (BVD6-24G2, rat IgG1; eBioscience, San Diego, CA ) together with 10  $\mu$ g of biotin-conjugated anti-mouse IFN- $\gamma$  (R4-6A2, rat IgG1; eBioscience, San Diego, CA). The antibodies were delivered in 125  $\mu$ L of endotoxin-free physiological saline (henceforth, “saline”), and were given to the animals at the end of the 14-day experimental period or, in the case of the zero-time control group, at 19 days of age immediately following the 24-h acclimation period. Half of the mice received 10  $\mu$ g of anti-mouse CD3 (145-2C11, Armenian hamster IgG; BD Bioscience, San Diego, CA) together with the biotin-conjugated anti-cytokine probe. After 4 h, blood samples were taken to permit analysis of the concentration of molecular complexes that had accumulated in the serum between the biotin-conjugated probes and their respective cytokines. The analyses were

performed using ELISA kits specific for mouse IL-4 and IFN- $\gamma$  (BD Bioscience, San Diego, CA). Finally, isotype control animals (n=2 per group) were included and each animal received 10  $\mu$ g of biotin-conjugated rat IgG1 (KLH/G1-2-2; Cedarlane Laboratories, Hornby, ON) by intraperitoneal injection in 125  $\mu$ L saline. Controls for mice stimulated with anti-CD3 (n=2 per group) received 10  $\mu$ g of Armenian hamster IgG (G235-2356; BD Bioscience, San Diego, CA) in the same saline-based injection.

### *2.8. Blood collection*

After measurement of body weight, mice were anesthetized with CO<sub>2</sub> and blood was taken from the orbital plexus of each animal as described previously [12,28]. The animals were then killed by cervical dislocation without recovering consciousness, and the blood was allowed to clot at room temperature for 30-45 minutes. The resulting serum was stored at -80°C.

### *2.9. Carcass analysis*

Carcasses were stored at -20°C to await analysis. Dry matter and lipid contents were determined as described elsewhere [18,21-23].

### *2.10. Statistical analyses*

The pre-determined upper limit of probability for statistical significance throughout this investigation was  $P \leq 0.05$ , and analyses were performed using the SAS system for Windows (version 8.2). Data from the two experiments involving multiple groups were subjected to either a one-way ANOVA or a two-way ANOVA and, in the

latter case, diet and T cell stimulus served as main effects. If justified by the resulting probability value (i.e.,  $P \leq 0.05$ ), multiple-group analyses were extended by application of Tukey's Studentized Range test. This was done within main effects in the case of a two-way ANOVA. Moreover, if a statistically significant interaction term emerged from a two-way ANOVA, the permissible pre-planned comparisons (i.e., equal in number to the treatment degrees of freedom) were made using the least squares means procedure. Data from the supplementary, two-group experiment were subjected to two-tailed Student's t-test. Throughout this investigation, data sets that failed to conform to a normal distribution according to each of the four tests applied by the SAS system, were transformed so as to comply with this underlying assumption of parametric testing. Where transformation attempts failed, data from experiments involving multiple groups were subjected to the Kruskal-Wallis test ( $\chi^2$  approximation) followed, if justified by the statistical probability outcome ( $P \leq 0.05$ ), by Wilcoxon two-sample testing. Nonparametric testing of data sets involving only two groups was accomplished by means of Wilcoxon two-sample comparisons.

### **3. Results**

#### *3.1. The 14-day malnutrition protocols elicited distinct weight loss pathologies*

Growth indices are shown in Table 1 and reflect similar outcomes in the separate studies. Initial body weights did not differ among groups, and the food intakes and gains in fat and lean tissue exhibited by the age-matched control group were comparable to previous results pertaining to C57BL/6J weanlings given free access to the same complete purified diet [12,19-21,25,29,30]. In addition, the malnutrition pathologies

produced in this investigation were comparable to the pathologies reported in previous studies demonstrating a Type 2 cytokine-polarized serum immunoglobulin profile [12] and depression in adaptive cell-mediated inflammatory competence [18,21,25] in the same experimental systems. Thus, weight loss did not differ between the two malnourished groups which exhibited deficits in both lean and fat tissue. However, the restricted intake protocol induced a greater loss of carcass lipid (and, hence, a greater decrement in carcass energy) than the low-protein protocol. Moreover, the food intakes of the malnourished groups (low relative to the intake of the age-matched control) differed from one another in a manner consistent with their differing carcass energy deficits. As discussed previously [29], the low-protein protocol elicited a wasting deficit of both protein and energy, whereas the restricted intake protocol produced mainly a deficit of energy.

*3.2. Anti-CD3 stimulation in vitro elicited a type 2-polarized cytokine response by mononuclear cells from lymph nodes and spleen of weanling mice in acute energy deficit but not in combined deficit of protein and energy: 14-day weight loss protocols*

Mean IFN- $\gamma$  concentrations in the mononuclear cell suspensions, independent of dietary group, were below the minimum detection limit (unstimulated groups), 307 pg/mL (anti-CD3 stimulus, only) and 556 pg/mL (combined anti-CD3 and anti-CD28 stimulation). Mean IL-4 concentrations in the same cell suspensions and for the same stimulus groups, likewise independent of diet, were 5.1, 39.2 and 42.3 pg/mL. Thus, independently of dietary group, plate-bound anti-CD3 increased the concentration of IL-4 and IFN- $\gamma$  detected in culture fluids of the mononuclear cell suspensions relative to the

cytokine levels supported by cultures not exposed to stimulation in vitro ( $P < 0.0001$  for both cytokines according to the Wilcoxon two-sample test). By contrast, inclusion of soluble anti-CD28 in the cultures exerted no statistically significant influence on the impact of anti-CD3 stimulation ( $P = 0.10$  and  $0.64$  for IFN- $\gamma$  and IL-4, respectively, according to the Wilcoxon two-sample test). The concentrations of IL-4 and IFN- $\gamma$  did not differ among dietary groups in culture fluids of mononuclear cells that were not subjected to polyclonal stimulation (results not shown,  $P = 0.21$  and  $0.60$ , respectively).

The models of acute malnutrition used herein can increase the percentage of T cells in the mononuclear cell populations of secondary lymphoid organs, although this phenomenon is seen only inconsistently in the food intake restriction model [19,20]. Consequently, the percentage of cells exhibiting a CD3 $\epsilon^+$  surface phenotype was assessed in the total population of mononuclear cells identified by forward-angle light scatter in the flow cytometer, and the outcome of this analysis is shown in Table 1. Relative to age-matched controls, the percentage of CD3 $\epsilon^+$  mononuclear cells was unaffected by the food intake restriction protocol, but was high in the mice fed the low-protein diet. No ontogeny was apparent in this index (age-matched group compared to zero-time control). In turn, the surface marker data were used to normalize the cytokine concentrations supported in vitro according to T cell numbers, and the resulting comparison among dietary groups is shown in Fig. 1. These results have been calculated by subtraction of the concentrations of cytokine found in cultures not subjected to polyclonal stimulation. On a per CD3 $\epsilon^+$  cell basis, the mononuclear cells recovered from the restricted intake group supported a higher concentration of IL-4, but a lower concentration of IFN- $\gamma$ , than the age-matched control group in response to polyclonal stimulation. By contrast, the

group fed the low-protein diet did not differ statistically from the age-matched control group in the concentration of either IL-4 or IFN- $\gamma$  found in culture fluids. No ontogeny was apparent in this index for either cytokine (age-matched control group compared to zero-time control). Moreover, in parallel with the findings expressed independently of T cell numbers, no effect of anti-CD28 stimulation was apparent over and above the response to plate-bound anti-CD3, alone, when the cytokine concentrations were expressed on a per T cell basis (results not shown,  $P > 0.05$  for each cytokine according to Tukey's Studentized Range test).

*3.3. Anti-CD3 stimulation in vivo elicited a type 2-polarized blood cytokine profile in weanling mice subjected to acute energy deficit but not in combined deficit of protein and energy: 14-day weight loss protocols*

In the absence of polyclonal anti-CD3 stimulation, the in vivo cytokine capture assay detected no differences related either to diet (comparisons among age-matched control, low-protein and restricted intake groups) or to ontogeny (comparison between age-matched control group and zero-time control) in the blood serum concentration of biotin-conjugated anti-cytokine complexes of either IL-4 ( $P = 0.56$ ) or IFN- $\gamma$  ( $P = 0.12$ ) that accumulated in the four-hour test period. Mean values of the four groups of mice (not shown) ranged from 0 - 4 pg/mL and from 0 - 47 pg/mL for IL-4 and IFN- $\gamma$ , respectively, when corrected for negative (isotype) control values which averaged 86 pg/mL (IL-4) and 57 pg/mL (IFN- $\gamma$ ). Stimulation with anti-CD3 increased the four-hour accumulation of both cytokine complexes in the blood serum independently of dietary

group ( $P < 0.0001$ , Kruskal-Wallis test). The comparison among dietary groups following stimulation with anti-CD3 is shown in Fig. 2. These results have been calculated by subtraction of negative (isotype) control values averaging 104 pg/mL (IL-4) and 73 pg/mL (IFN- $\gamma$ ). No ontogeny was apparent in this index for either cytokine (zero-time control group compared to age-matched control). The restricted intake group exhibited a low level of IFN- $\gamma$  accumulation in the serum relative to both age-matched controls and the zero-time group, but did not differ from these groups in terms of IL-4 complex accumulation. The group given the low-protein diet did not differ from the age-matched control group in terms of four-hour accumulation of either cytokine complex in the blood serum, but exhibited an elevated serum concentration of IL-4 complex relative to the zero-time controls. The restricted intake group exhibited a low serum level of both cytokine complexes relative to the group fed the low-protein diet.

#### *3.4. Anti-CD3 stimulation in vivo elicited a type 2-polarized blood cytokine profile in weanling mice subjected to combined protein and energy deficit: 26-day weight loss protocol*

It was unclear whether the difference in cytokine profile between the 14-day low-protein and restricted intake protocols reflected their fundamental metabolic disparities or simply their differing degrees of wasting as apparent in energy decrements. Hence, a supplementary experiment was performed in which the low-protein diet was fed to weanling C57BL/6J mice, initially 19 days old, with a view to achieving an energy decrement similar to that routinely observed in mice subjected to the 14-day restricted intake protocol. The experimental period was extended to 26 days during which the mice

were given free access to either the complete purified diet or the isocaloric low-protein formulation. Performance indices are shown in Table 2. Initial body weights did not differ between the two groups, and the low-protein diet exerted the anticipated influence on the indices that were assessed. In particular, although formal statistical comparison is precluded, the mean carcass fat content of the 26-day low-protein group (Table 2) declined far below the mean values shown in Table 1 for the weanling animals subjected to the same low-protein diet for only 14 days. Unsurprisingly, therefore, the 26-day low-protein protocol fulfilled its primary purpose of imposing a carcass energy decrement exceeding that achieved when the same nitrogen-deficient diet was fed for the shorter 14-day experimental period. Under these circumstances, the *in vivo* cytokine capture assay revealed low serum concentrations of biotin-conjugated anti-IFN- $\gamma$  complex in the malnourished group relative to age-matched controls 4 h after challenge with anti-CD3 *in vivo*, whereas no statistically significant diet-related effect was apparent, in the same animals, on the serum concentration of anti-IL-4 complex (Fig. 3). The results shown in Figure 3 were calculated by subtracting negative (isotype) control values averaging 27 pg/mL and 17 pg/mL for IL-4 and IFN- $\gamma$ , respectively.

It is pointed out elsewhere [12,21,23,26,29,30] that the low-protein model of dietary imbalance used herein mimics essential features of incipient kwashiorkor, whereas the restricted intake protocol reproduces critical features of marasmus. As expected of a model of incipient kwashiorkor [12,21,23,26,29,30], the carcass fat decrement of the 26-day low-protein model did not reach the extent of loss seen in the 14-day restricted intake system, at least according to inspection of the results shown in Tables 1 and 2. Nevertheless, the key outcome is that, assessed *in vivo*, the polarizing

cytokine profile of the T cell compartment in the low-protein pathology (Fig. 3) was indistinguishable from that of the restricted intake pathology (Fig. 2) when a systemic energy loss of sufficient duration (26 d), hence magnitude, was imposed.

**Table 1**

**Performance outcomes, including critical composition characteristics, of weanling mice at 19 days of age or after 14-day experimental protocols initiated at 19 days of age<sup>1</sup>**

Index	Dietary Group <sup>2</sup>				SEM
	B	C	LP	R	
<u>Experiment 1: Cytokine Production In Vitro</u>					
Initial body weight (g/mouse)	8.3	8.3	8.6	8.1	0.25
Final body weight (g/mouse) <sup>3</sup>	---	16.1 <sup>A</sup>	6.4 <sup>B</sup>	6.3 <sup>B</sup>	0.03
Food intake (g/mouse · 14 d) <sup>3</sup>	---	46.4 <sup>A</sup>	18.6 <sup>B</sup>	11.7 <sup>C</sup>	0.03
Food intake (g/g body weight · d) <sup>3</sup>	---	0.27 <sup>A</sup>	0.18 <sup>B</sup>	0.12 <sup>C</sup>	0.04
Carcass dry matter (g/100g wet weight)	29.7 <sup>AB</sup>	31.2 <sup>A</sup>	27.7 <sup>BC</sup>	26.4 <sup>C</sup>	0.57
Carcass lipid (g/100g wet weight) <sup>4</sup>	8.6 <sup>A</sup>	8.5 <sup>A</sup>	4.8 <sup>B</sup>	2.2 <sup>C</sup>	---
% CD3 <sup>+</sup> mononuclear cells in spleen and nodes <sup>5</sup>	18.9 <sup>C</sup>	29.9 <sup>BC</sup>	44.7 <sup>A</sup>	39.0 <sup>AB</sup>	3.36
<u>Experiment 2: Cytokine Production In Vivo</u>					
Initial body weight (g/mouse) <sup>6</sup>	8.3	8.6	8.8	8.6	2.97
Final body weight (g/mouse) <sup>3</sup>	---	17.9 <sup>A</sup>	6.7 <sup>B</sup>	6.4 <sup>B</sup>	0.02
Food intake (g/mouse · 14 d) <sup>3</sup>	---	65.1 <sup>A</sup>	21.2 <sup>B</sup>	11.5 <sup>C</sup>	0.03
Food intake (g/g body weight · d) <sup>3</sup>	---	0.26 <sup>A</sup>	0.13 <sup>B</sup>	0.07 <sup>C</sup>	0.03
Carcass dry matter (g/100 g wet weight) <sup>7</sup>	28.1 <sup>B</sup>	32.1 <sup>A</sup>	26.5 <sup>C</sup>	26.3 <sup>C</sup>	---
Carcass lipid (g/100 g wet weight) <sup>3</sup>	8.0 <sup>A</sup>	9.2 <sup>A</sup>	4.8 <sup>B</sup>	2.7 <sup>C</sup>	0.05

<sup>1</sup>Mean values. Within a row, values not sharing a superscript letter differ ( $P \leq 0.05$ ) according to Tukey's Studentized Range test or according to the Kruskal-Wallis procedure followed by Wilcoxon two-sample comparisons.

<sup>2</sup>B: zero-time control, 19 d old; C: group that consumed complete diet ad libitum; LP: group that consumed low-protein diet ad libitum; R: group that consumed complete diet in restricted daily quantities.

<sup>3</sup>From ANOVA of natural log-transformed data. Mean values are antilogs of log means.

<sup>4</sup>Kruskal-Wallis test of Wilcoxon rank sums which were as follows: B, 247.0; C, 243.0; LP, 160.5; R, 90.5.

<sup>5</sup>Percentage of cells expressing CD3 $\epsilon$  in the total mononuclear cell population identified by forward-angle light scatter in the flow cytometer.

<sup>6</sup>From ANOVA of square-transformed data. Mean values are square roots of squared means.

<sup>7</sup>Kruskal-Wallis test of Wilcoxon rank sums which were as follows: C, 880.0; B, 535.5; LP, 379.5; R, 285.0.

**Table 2****Performance outcomes, including critical composition characteristics, of weanling mice at 19 days of age or after 26-day low-protein dietary protocol initiated at 19 days of age<sup>1</sup>**

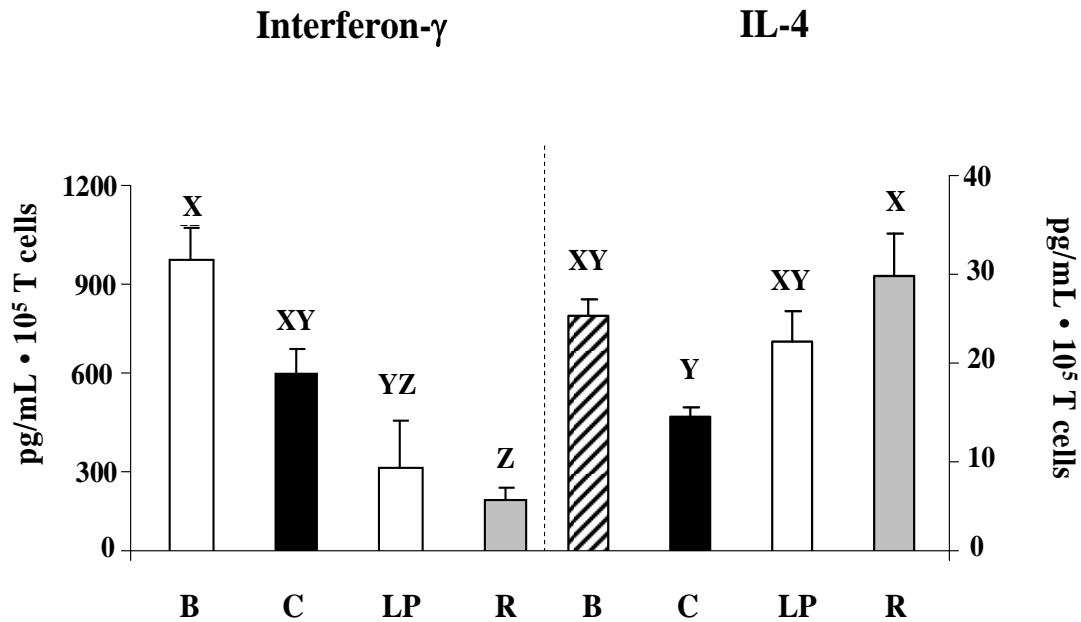
Index	Dietary Group <sup>2</sup>	
	C	LP
Initial body weight (g/mouse) <sup>3</sup>	8.4 (8.0 - 9.0)	8.4 (8.1 - 9.1)
Final body weight (g/mouse)	20.7 ± 1.84	6.1 ± 0.43 <sup>A</sup>
Food intake (g/mouse · 26 d)	79.0 ± 10.15	29.8 ± 4.53 <sup>A</sup>
Food intake (g/g body weight · d)	0.21 ± 0.033	0.16 ± 0.026 <sup>A</sup>
Carcass dry matter (g/100g wet weight) <sup>4</sup>	34.1 (31.8 - 36.4)	26.8 (19.8 - 29.8) <sup>A</sup>
Carcass lipid (g/100g wet weight)	10.4 ± 1.01	3.7 ± 0.50 <sup>A</sup>

<sup>1</sup>Mean values (range, or ± standard deviation). Within a row, superscript letter “A” signifies statistical difference ( $P \leq 0.05$ ) from age-matched control group (designated “C”) according to two-tailed Student’s t-test or according to Wilcoxon two-sample comparison.

<sup>2</sup>C: group that consumed complete diet ad libitum; LP: group that consumed low-protein diet ad libitum.

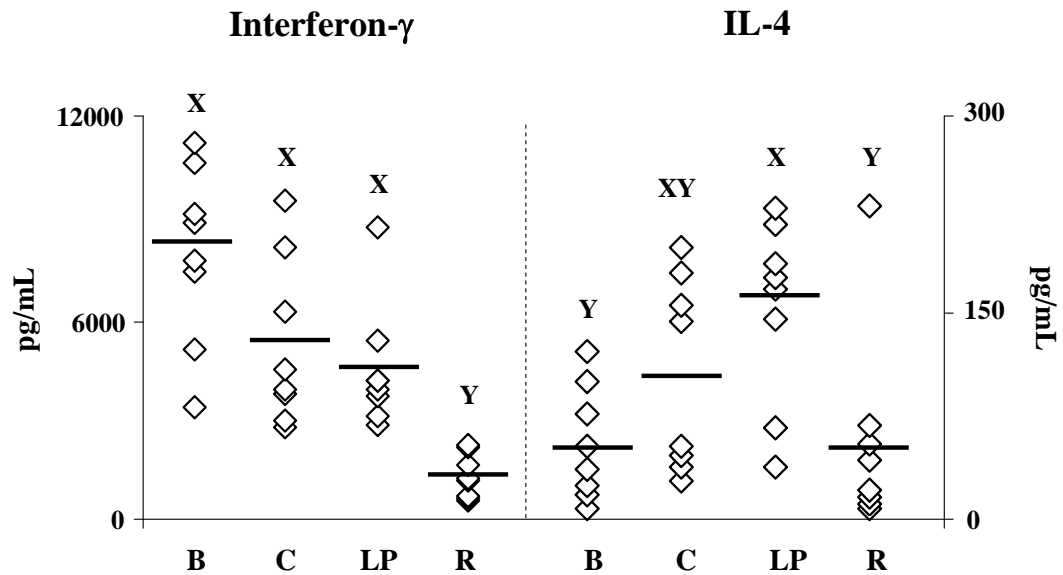
<sup>3</sup>Wilcoxon rank sums were 154.0 and 146.0 for groups C and LP, respectively.

<sup>4</sup>Wilcoxon rank sums were 222.0 and 78.0 for groups C and LP, respectively.



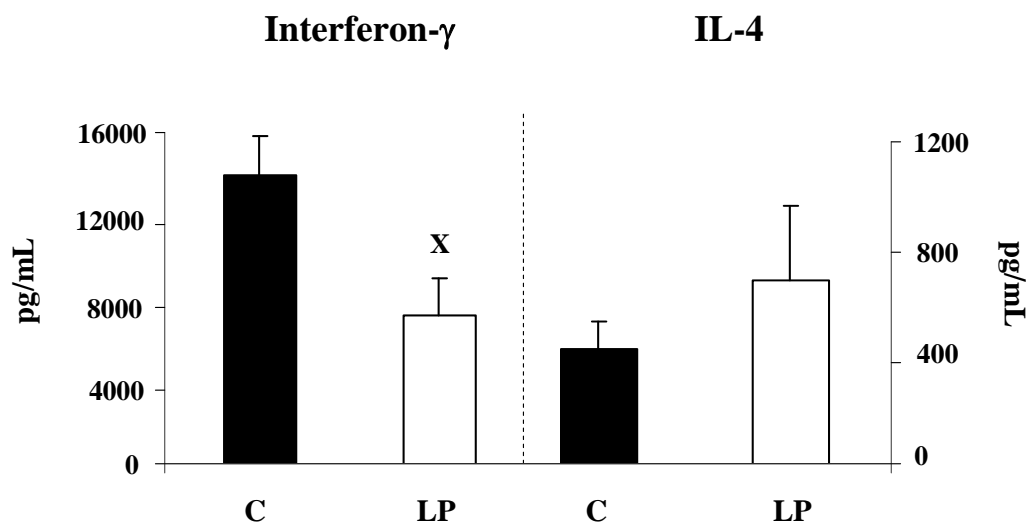
**Fig. 1.** Concentration of interferon(IFN)- $\gamma$  and IL-4 found in cultures of mononuclear cells from the spleen and lymph nodes after 24 h in vitro. Each culture included  $2 \times 10^5$  viable cells and polyclonal stimulation was achieved with plate-bound anti-CD3 (145-2C11, 5  $\mu$ g/mL) and, in some cultures, soluble anti-CD28 (37.51.1, 20  $\mu$ g/mL). Malnourished mice were given free access to a purified low-protein diet (group LP, n=10) or were fed a complete purified diet in restricted daily quantities (group R, n=8) to lose, in either case, 1.5 – 2% of initial body weight daily during a 14-d experimental period. Age-matched controls (group C, n=10) were permitted free access to the purified complete diet for the same length of time, and 19-d-old zero-time controls (group B, n=10) were included. Male and female C57BL/6J mice were used in equal numbers in each of the four experimental groups. Each sample of malnourished animals was made

by pooling 2-4 mice (group LP) or 2-8 mice (group R), and each pooled sample was assigned a single degree of freedom for the purpose of statistical analysis. Bars represent means and SEM values are shown. Within each cytokine, data from stimulated cultures were corrected by subtraction of the cytokine level found in unstimulated cultures of the same dietary group and were normalized on a per  $10^5$  T cell basis. These data sets were subjected to two-way ANOVA (main effects: diet and type of stimulus, i.e. anti-CD3  $\pm$  anti-CD28). Means are antilogs of log-transformed means and diet main effect P values were 0.002 (IFN- $\gamma$ ) and 0.05 (IL-4). Bars not sharing an upper case letter differ ( $P \leq 0.05$ ) according to Tukey's Studentized Range test, and pooled SEM was 0.336 and 0.195 for IFN- $\gamma$  and IL-4, respectively. The diet effect was independent of stimulus (interaction  $P=0.95$  and  $0.65$  for IFN- $\gamma$  and IL-4, respectively). The stimulus main effect (not shown) was statistically significant for both cytokines ( $P < 0.0001$ ), but anti-CD28 stimulation elicited no further production of either IFN- $\gamma$  or IL-4 beyond the response to anti-CD3, alone ( $P > 0.05$ , Tukey's Studentized Range test).



**Fig. 2.** Concentrations of IFN- $\gamma$  and IL-4 complexed with their respective biotin-conjugated anti-cytokine antibody (R4-6A2 and BVD6-24G2) in the serum of C57BL/6J mice four hours after i.p. injection of the biotin-tagged probes together with 10 $\mu$ g/mouse of anti-CD3 (145-2C11). Malnourished mice were given free access to a purified low-protein diet (group LP) or were fed a complete purified diet in restricted daily quantities (group R) to lose, in either case, 1.5 – 2% of initial body weight daily during a 14-d experimental period. Age-matched controls (group C) were permitted free access to the purified complete diet for the same length of time, and 19-d-old zero-time controls (group B) were included. Eight mice (four males and four females) were included in each of the four experimental groups within each cytokine. Each point in the dot-plot represents one animal but, occasionally, overlapping data points obscure the sample size of eight in a group. Each data point was corrected by subtraction of the mean value of the isotype control, and the mean of each group is depicted as a horizontal bar inserted within the

column of points. Within each cytokine, groups not sharing an upper case letter differ ( $P \leq 0.05$ ) according to Wilcoxon two-sample comparisons. Kruskal-Wallis rank sums were 191, 131, 139 and 67 (groups B, C, LP and R, respectively,  $P=0.01$ ) for the data pertaining to the serum IFN- $\gamma$  complex and were 110, 135, 190 and 93 (groups B, C, LP and R, respectively,  $P=0.05$ ) for the data pertaining to the serum IL-4 complex.



**Fig. 3.** Concentrations of IFN- $\gamma$  and IL-4 complexed with their respective biotin-conjugated anti-cytokine antibody (R4-6A2 and BVD6-24G2) in the serum of C57BL/6J mice four hours after i.p. injection of the biotin-tagged probes together with 10 $\mu$ g/mouse of anti-CD3 (145-2C11). Malnourished mice were given free access to a purified low-protein diet (group LP) during a 26-d experimental period. Age-matched controls (group C) were permitted free access to the purified complete diet for the same length of time. Six mice (three males and three females) were included in each experimental group. Bars represent mean values and SEM is shown for each mean. Within each cytokine, data were corrected by subtraction of the mean value of the isotype control. The letter “X” signifies statistically significant difference from group C ( $P \leq 0.05$ ) according to Student’s two-tailed t-test.

#### 4. Discussion

This investigation reveals a shift toward Type 2 cytokine polarization within the effector/memory T cell compartment of metabolically dissimilar murine models of acute (i.e., wasting) pre-pubescent malnutrition. As discussed elsewhere [12,21,23,26,29,30], the two experimental systems used herein are relevant to the distinct and well-defined human pediatric malnutrition pathologies of marasmus and kwashiorkor. In particular, the low-protein protocol elicits the hallmark features of kwashiorkor, i.e. fatty liver and edema, but only if extended beyond four weeks [31]. Hence, the two low-protein models used herein are best understood as models of incipient kwashiorkor. By contrast, as confirmed in this investigation, the restricted intake model exhibits no edema or fatty liver but is marked, as would be expected of a marasmic system, by extreme carcass lipid decrement exceeding even that of the models of imminent kwashiorkor [23]. The phenomenon that has emerged appears confined to the advanced stages of these pathologies, is independent of sex, and reflects complementary findings in vitro and in vivo following application of physiologically relevant polyclonal stimulation through the T cell receptor. Moreover, although Type 2 cytokine polarity is characteristic of murine neonatal effector/memory T cells [32], inclusion of a zero-time control group in the experimental design revealed that the malnutrition-associated phenomenon represents more than a biologically trivial delay in ontogeny. The outcome of the present investigation reveals a restructured cytokine polarity within the effector/memory T cell compartment in support of the non-inflammatory adaptive immune competence classically characteristic of acute pre-pubescent deficits of protein and energy [2].

A cytokine response attributable to the effector/memory T cell compartment was isolated for study both in vitro and in vivo in this investigation. This is apparent both on the basis of the timeframes within which responses were assessed and on the basis of the stimulant that proved sufficient to elicit a response. First, to achieve effector status, naïve T cells require more than the short response periods studied in the present investigation [8,9]. Secondly, naïve T cells cannot differentiate to effector status if stimulated only through the T cell receptor [10]. However, in the present investigation, the in vivo response was elicited exclusively by stimulation through the CD3 molecule and, in vitro, co-stimulation through CD28 failed to increase the production of end-stage cytokines. The resulting unambiguous discernment of the cytokine polarity of the effector/memory T cell compartment was essential to the core objective of this investigation.

No in vivo index of cytokine production can be independent of physiological turnover, a potentially crippling confounder in relation to blood cytokines because of their brief half-lives that are generally measured in minutes [33-36]. The in vivo cytokine capture assay traps newly-synthesized cytokine in an antibody complex that extends the half-life of the bound cytokine to hours or days in the blood of the mouse [34,36-39]. Therefore, when performed with a suitably short assessment period, e.g. 4 h as in the present investigation, the assay is not overwhelmed by the factor of turnover and yields an index relating to net systemic cytokine production rate in vivo. In fact, this assay strategy is simply an extension of the physiological phenomenon whereby the clearance of cytokines from the blood is retarded when these molecules form complexes with soluble cytokine receptors [40] or anti-cytokine autoantibodies [41]. In the context of the present investigation, the cytokine capture index may be regarded as the in vivo

cytokine signature of the effector/memory T cell compartment. By contrast, the in vitro type of assay used herein yields results that may relate more directly to T cell cytokine production but, because of the unphysiological context of a cell culture microenvironment, these results serve best in a supplementary capacity to facilitate interpretation of the in vivo capture assay. In this context, it should be noted that mononuclear cells from both mucosal and deep-tissue lymphoid organs were included in the in vitro assays because of their differing Type 1 and Type 2 cytokine profiles [42]. However, it is unlikely that a sample representative of proportions within the intact animal was obtained and it is particularly important, therefore, that the in vivo and in vitro indices pursued in this investigation yielded complementary and consistent immunological perspectives. Taken together, the indices of function selected for this investigation have provided insight into the influence of acute forms of pre-pubescent protein and/or energy deficit on the Type 1 and Type 2 polarizing cytokine signature of the effector/memory T cell compartment in vivo.

A simple interpretation of the present investigation is that a Type 2 cytokine-polarized T cell compartment develops in metabolically dissimilar forms of acute pre-pubescent malnutrition but, being evident in the low-protein model only in its extended (26-d) form, this immunological characteristic appears confined to the most advanced stages of energy loss. This interpretation also accommodates a previous report [14] in which acute combined protein and energy deficit produced Type 2 cytokine polarization in the adult mouse according to an in vitro assessment of splenic T cells in which the effector/memory compartment was not isolated for study. Thus, in the metabolic setting of acute malnutrition as revealed here and elsewhere [14], Type 2 cytokine polarity

appears to develop within the T cell compartment because the ability to produce IFN- $\gamma$  declines while the capacity to produce IL-4 is preserved. Additional findings consistent with this conclusion have been obtained recently regarding the capacity of the effector/memory T cell compartment, in the experimental systems used herein, to produce the important anti-inflammatory type 2 cytokine, IL-10 (Monk and Woodward, unpublished). Further, this shift in T cell end-stage cytokine polarity may be sustained in the face of concurrent infection. Although blood lymphocytes cannot be taken as representative of the systemic pool [43], blood mononuclear cells from acutely malnourished children suffering various gastrointestinal and respiratory infections exhibited reduced expression of IFN- $\gamma$  mRNA coupled with an elevated expression of IL-4 message [44]. In the same clinical setting, children with diverse mucosal infections and various degrees and forms of acute malnutrition exhibited a reduced proportion of blood T cells expressing IFN- $\gamma$  following stimulation in vitro while the proportion of cells expressing IL-4 remained unaffected [45]. A large body of clinical and experimental evidence, summarized elsewhere [2], emphasizes the immunological similarity among metabolically diverse forms of acute malnutrition. This body of evidence continues to grow [12,23,30], and the present investigation adds new substance to it.

An interesting perspective emerges when the outcome of the present investigation is considered together with findings pertaining to stunting forms of pre-pubescent malnutrition. Unlike acute forms of protein and energy deficit, stunting produced by means of nitrogen-deficient diets usually depresses humoral competence in rodents while inflammatory cell-mediated immunity remains unaffected or may even be enhanced [46]. Similarly, mice stunted through restricted consumption of a complete diet exhibited

reduced Type 2 cytokine-dependent humoral protection against a nematode infection while the Type 1 cytokine-dependent cell-mediated response to the infecting organism was preserved [47]. Preservation of inflammatory cell-mediated competence is also common in stunted children [2]. Each of the differing murine models of pre-pubescent stunting, whether based on nitrogen-deficient dietary imbalance [48,49] or on restricted intake of a complete diet [47], elicits movement toward Type 1 cytokine polarity, discerned in vitro, within the T cell compartment of secondary lymphoid organs. Thus, the polarizing cytokine profile of the T cell compartment appears to differ between acute and chronic forms of pre-pubescent protein and energy deficit in their advanced stages; however, this index reflects reliably the balance between end-stage humoral and cell-mediated adaptive immune competence in these diverse malnutrition pathologies.

It is worthwhile to consider the importance of a re-configured effector/memory T cell compartment in relation to the classic immunological characteristic of acute malnutrition [2] in which inflammatory cell-mediated competence is consistently depressed while humoral responses are affected inconsistently and unpredictably. Our 14-day model of incipient kwashiorkor exhibits precisely this adaptive immune competence profile [18] at a stage of energy decrement that, according to the present investigation, precedes the emergence of a Type 2-polarized T cell compartment. Therefore, an altered balance in the polarizing cytokine profile of the T cell system undoubtedly supports and augments the non-inflammatory character of immune competence in advanced acute malnutrition, but is unlikely to be important in initiating the development of this distinctive immunological trait. As a further point of perspective, the outcome of the present investigation lends new support to the proposition

[2,11,12,30] that malnutrition-associated depression in inflammatory adaptive immune competence is a regulated pathophysiology incompatible with the widely-accepted model of metabolic disintegration.

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## Chapter 4

**Blood corticosterone concentration reaches critical illness levels early during acute malnutrition in the weanling mouse.**

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**Key words:** corticosterone; blood; energy deficiency; protein deficiency; mice

*The following chapter appears in its entirety in compliance with the formatting requirements for publication in Experimental Biology and Medicine.*

## **Abstract**

Acute (i.e., wasting) pediatric malnutrition consistently elevates blood glucocorticoid levels, but neither the magnitude of the rise in concentration, nor its kinetics, are clear. Male and female C57BL/6J mice, initially 19 days old, and CBA/J mice, initially 23 days old, consumed a complete purified diet either ad libitum (age-matched control) or in restricted daily quantities (mimicking marasmus), or they consumed a purified isocaloric low-protein diet ad libitum (mimicking incipient kwashiorkor). Serum levels of corticosterone were assessed by double antibody radioimmunoassay after 3, 6 and 14 days (C57BL/6J strain), or after 6 and 14 days in the genetically distant CBA/J strain. Age-matched control groups of both strains exhibited mean corticosterone levels of 5-30 ng/ml, whereas the acutely malnourished groups exhibited mean levels of this hormone that were elevated by more than an order of magnitude as early as 3 days after initiation of weight loss. This outcome was confirmed in a second experiment in which the serum corticosterone level of C57BL/6J weanlings was examined by competitive binding enzyme immunoassay 3 and 14 days after initiation of the dietary protocols. Therefore, deficits of protein and/or energy in weanling murine systems relevant to acute pediatric malnutrition elicit early elevations in blood glucocorticoid levels to a magnitude reminiscent of critical illness and multiple trauma. The key to this novel finding was an exsanguination method that permitted accurate assessment of the blood corticosterone level of the healthy, quiescent mouse. Overall, the results of this investigation provide a new perspective on the glucocorticoids as part of the early hormonal response to acute weanling malnutrition coincident with the shift toward catabolic metabolism and the initiation of depression in cellular immune competence.

## **Introduction**

The physiological response to acute deficits of protein and energy is orchestrated by endocrine hormones, and conserves substrates and energy while promoting glucose homeostasis and a metabolic preference for fat (1). Current understanding of this phenomenon is based mainly on the blood hormonal profile, a consistent feature of which is a high level of glucocorticoids. Although these hormones, specifically cortisol in humans and corticosterone in rodents, occupy a central position in the attempt to adapt to weight loss (1), the wasting-associated elevation in blood glucocorticoid concentrations is surprisingly ill-defined. Early studies revealed that blood levels of both total (2-6) and unbound (3, 5) cortisol are high in acutely malnourished children. However, both the magnitude of the increase in blood glucocorticoid levels and its time-kinetics remain unclear. Blood cortisol and corticosterone concentrations between 150 and 600 ng/ml are reported both in acutely protein and energy malnourished children (2-6) and in acutely malnourished young adult rodents (7, 8). The cited studies document malnutrition-associated glucocorticoid levels ranging from 1.3- to 6-fold the concentrations found in appropriately matched healthy subjects (80-190 ng/ml), with only one outcome (5) exceeding 3-fold. These results represent the full range of increases that have been reported in connection with acute malnutrition. Recently, however, the blood corticosterone level of the healthy, quiescent adolescent mouse was shown to lie between only 10 and 20% of the previously-accepted range (9). Thus, inflated quiescence reference levels may obscure the magnitude of the glucocorticoid response reported, to date, in acutely malnourished rodents and (by extrapolation) humans. In addition, the literature is scant and conflicting with regard to the time kinetics of this hormonal

response during acute malnutrition. One report could be interpreted to suggest that the glucocorticoid response constitutes an early component of the attempt to adapt to acute protein and energy deficit in the rodent (8). However, this remains only a point of logic, and a second report pertaining to rodents (7) did not concur. The objective of this investigation was to determine the magnitude and time kinetics of the rise in blood corticosterone concentration in relevant and metabolically diverse murine models of acute, weanling protein and energy deficit. An additional component of the objective was to eliminate the confounder of infection which can be a major contributor to the high blood levels of glucocorticoids in acute pediatric malnutrition (4).

## **Materials and Methods**

**Animals and Facilities.** Male and female mice were used from in-house breeding colonies of C57BL/6J and CBA/J strains. These colonies are maintained under conventional conditions, but incoming air is filtered, each cage is supplied with a filter lid and positive pressure is maintained relative to the adjoining corridor. According to examination of sentinel mice maintained in cages without filter lids, the colonies are free of common pathogens and intestinal parasites. Housing conditions were exactly as described previously (9-13), and the investigation was approved by the Animal Care Committee of the University of Guelph according to the guidelines of the Canadian Council on Animal Care.

**Diets and Feeding Protocols.** C57BL/6J mice were weaned at 18 days of age and acclimated for one day with free access to the complete purified diet described elsewhere (10, 11). Likewise CBA/J mice were weaned at 21 days of age and acclimated for two days. At 19 days of age (C57BL/6J) or 23 days of age (CBA/J), each mouse was randomly allocated to one of three experimental groups, namely an age-matched control group that consumed the complete diet ad libitum, a group that consumed the complete diet in restricted daily quantities, and a group given free access to a low-protein purified diet. The quantity of diet given the mice of the restricted intake group was calculated daily as described previously by this laboratory (11) with a view to achieving a loss of 1.5-2% of initial body weight per day throughout the experimental period. As described elsewhere (10), the purified low-protein diet contained 0.6% crude protein (as fed) and was prepared by replacement of most of the egg white (80% crude protein; U.S. Biochemical, Cleveland, OH) of the complete diet (18% crude protein) with an equal weight of cornstarch (St. Lawrence Starch, Port Credit, Canada). All animals had free access to clean tap water and coprophagy was permitted.

**Experimental Design.** Two experiments were performed. In the first experiment, cohorts of C57BL/6J weanlings were examined after 3, 6, or 14 days (n= 4 males and 4 females per dietary group at each time point, except n=5 males and 6 females at day 14), and cohorts of CBA/J weanlings were examined after 6 and 14 days (n=3 males and 3 females per dietary group at each time point). In the second experiment, cohorts of C57BL/6J weanlings were examined after either 3 or 14 days (n=2 males and 3 females per dietary group at each time point). The mice were housed individually

throughout acclimation and experiment, and interaction with the animals was limited to the period between 0900 and 1100 h. At the end of the pre-determined experimental period for a cohort of animals, blood was taken from each mouse which was then killed and weighed without recovering consciousness.

**Blood Collection.** Blood was taken from the orbital plexus of each mouse under CO<sub>2</sub> anesthesia following a protocol designed to minimize acute pre-anesthesia stress (9). Samples were allowed to clot at room temperature for up to 60 minutes, and the resulting serum was stored at -80°C.

**Serum Corticosterone Assay.** Serum total corticosterone concentration was determined by double antibody radioimmunoassay (Immuchem <sup>125</sup>I Corticosterone Kit; ICN Biomedicals, Inc., Costa Mesa, CA) in the first experiment and by competitive binding enzyme immunoassay (OCTEIA Corticosterone Kit; Medicorp [Immunodiagnostic Systems, Ltd.], Montreal, P.Q.) in the second study. Both assays were performed according to the manufacturer's instructions and permitted use of unextracted serum in small volumes (10–30 µl) without protein denaturation. The outcome of the radioimmunoassay was based on collection of 10,000 counts per sample except for the background which was assessed on the basis of 25-minute counts to be 156 ± 5 cpm (mean ± SD). In this assay, nonspecific binding did not exceed 2.4% of total counts, while within-assay coefficient of variation was 2% (n=20) and between-assay coefficient of variation was 9% (n=2). The lower detection limit of the assay, estimated as described elsewhere (14), was 2.6 ng/ml and the standard curve was linear to a

concentration of at least 1000 ng/ml. Likewise the intra- and inter-assay coefficients of variation for the enzyme immunoassay were 2.7% (n=6) and 10.6% (n=2), respectively, and the detection limit was 0.4 ng/ml.

**Carcass Analysis.** Carcasses were stored at  $-20^{\circ}\text{C}$  to await assay of dry matter and lipid contents as described elsewhere (10-13).

**Statistical analysis.** The SAS system for windows (version 8.2) was used for statistical analysis (15) and a predetermined upper limit of probability of  $P\leq 0.05$  was applied for statistical significance. Data were subjected to one-way ANOVA (thereby treating each cohort, i.e. time point, independently) followed, if justified by the resulting statistical probability (i.e.,  $P\leq 0.05$ ), by Tukey's Studentized Range test. Data sets not exhibiting a normal distribution according to each test applied by the SAS program ( $P\leq 0.05$ ) were transformed to normality by either square root or logarithmic conversion. Bartlett's test was applied to assess homogeneity of variances.

## **Results**

Growth indices pertaining to the C57BL/6J weanlings examined at days 3, 6 and 14 in the first experiment are shown in Table 1. Within each time period, initial body weights did not differ among dietary groups, and the age-matched control group exhibited gains of fat and lean tissue that were comparable to outcomes reported previously (11, 12). Likewise, the loss of lean and fat tissue imposed by the malnutrition protocols was similar to that observed previously and revealed, as would be predicted

from the food intakes, that the restricted intake protocol imposed a greater loss of energy than the low-protein procedure (11, 12). Thus, the results were consistent with previous studies in which the restricted intake and low-protein protocols produced acute weanling pathologies mimicking, and relevant to, marasmus and incipient kwashiorkor, respectively (13). Similar pathologies were produced in the CBA/J strain weanlings subjected to the same acute nutritional deficits (results not shown).

Serum corticosterone concentrations of the C57BL/6J weanlings in the first experiment are shown in Figure 1a. The levels of the age-matched control groups averaged between 5 and 20 ng/ml and, thus, were comparable to the concentration reported elsewhere (9) as typical of the quiescent adolescent mouse. The mean serum corticosterone level induced by each of the malnutrition protocols was 17-fold that of the age-matched controls as early as three days after the initiation of weight loss. The CBA/J strain yielded a comparable outcome (statistics not shown) with respect to both the serum corticosterone concentration of the age-matched control groups (20-30 ng/ml) and the magnitude of the hormonal response to the two acute deficiency pathologies (350-700 ng/ml). Sex effects were not apparent so that, in all cases, the results were combined accordingly for statistical analysis.

A small confirmatory investigation was conducted to determine whether the outcome of the first experiment might be dependent on the hormonal assay technique. Growth indices (not shown) revealed comparable pathologies to those of the first experiment at both early (day 3) and advanced (day 14) stages of weight loss in both models of acute malnutrition. Likewise, assay of serum corticosterone levels by competitive binding enzyme immunoassay confirmed the previous findings (based on

radioimmunoassay) with respect to both the concentration expected of healthy adolescent animals (5-15 ng/ml) and the speed and magnitude of malnutrition-associated rise in hormone concentrations (Figure 1b).

Table 1. Experiment 1: Initial and final body weights, food intakes and carcass compositions of C57BL/6J strain mice<sup>a</sup>

Index	Dietary Group <sup>b</sup>			SEM
	C	LP	R	
<u>3-day Experimental Period</u>				
Initial body weight (g)	8.3	8.8	8.5	0.21
Final body weight (g)	10.0 <sup>A</sup>	7.8 <sup>B</sup>	7.8 <sup>B</sup>	0.76
Food intake (g) <sup>c,d</sup>	8.9 <sup>A</sup>	5.2 <sup>B</sup>	3.9 <sup>C</sup>	0.06
Carcass composition (% wet weight)				
Dry matter	27.3 <sup>A</sup>	30.0 <sup>AB</sup>	31.2 <sup>B</sup>	0.92
Lipid <sup>e</sup>	6.8 <sup>A</sup>	4.6 <sup>AB</sup>	3.5 <sup>B</sup>	0.19
<u>6-day Experimental Period</u>				
Initial body weight (g) <sup>d</sup>	8.7	8.3	8.1	0.04
Final body weight (g) <sup>d</sup>	12.4 <sup>A</sup>	6.9 <sup>B</sup>	7.1 <sup>B</sup>	0.07
Food intake (g)	20.5 <sup>A</sup>	11.0 <sup>B</sup>	5.9 <sup>C</sup>	1.38
Carcass composition (% wet weight)				
Dry matter	28.9	27.6	28.0	0.64
Lipid <sup>d</sup>	6.0 <sup>A</sup>	3.3 <sup>B</sup>	2.5 <sup>C</sup>	0.06
<u>14-day Experimental Period</u>				
Initial body weight (g) <sup>d</sup>	8.8	8.9	8.5	0.02
Final body weight (g) <sup>e</sup>	17.7 <sup>A</sup>	5.9 <sup>B</sup>	6.1 <sup>B</sup>	0.28
Food intake (g)	50.7 <sup>A</sup>	25.1 <sup>B</sup>	13.3 <sup>C</sup>	1.87
Carcass composition (% wet weight)				
Dry matter	32.5 <sup>A</sup>	29.2 <sup>B</sup>	26.7 <sup>B</sup>	0.83
Lipid <sup>d</sup>	7.0 <sup>A</sup>	4.2 <sup>B</sup>	2.4 <sup>C</sup>	0.09

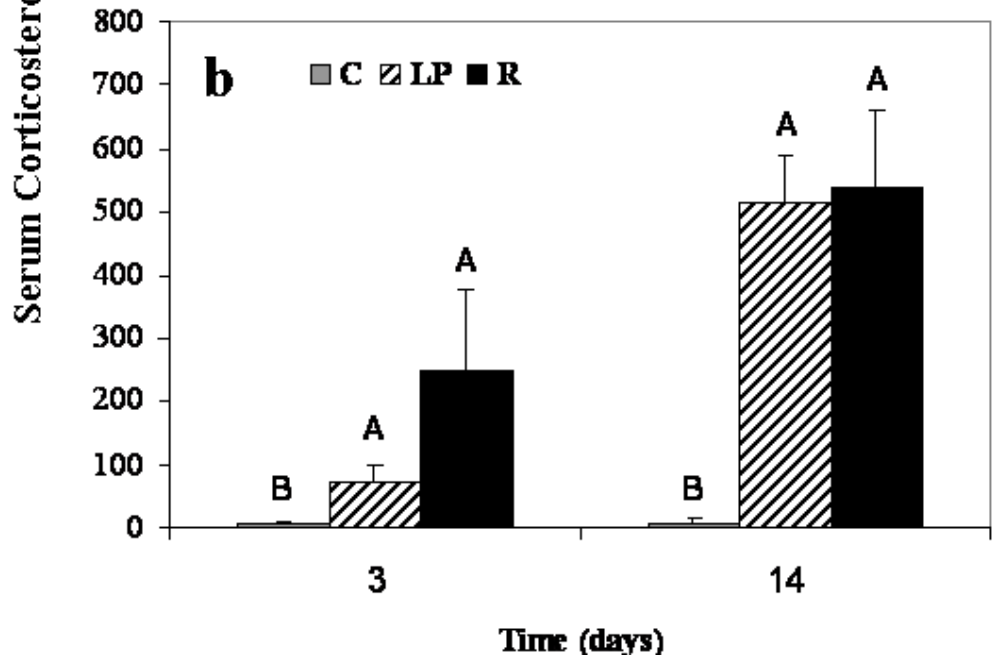
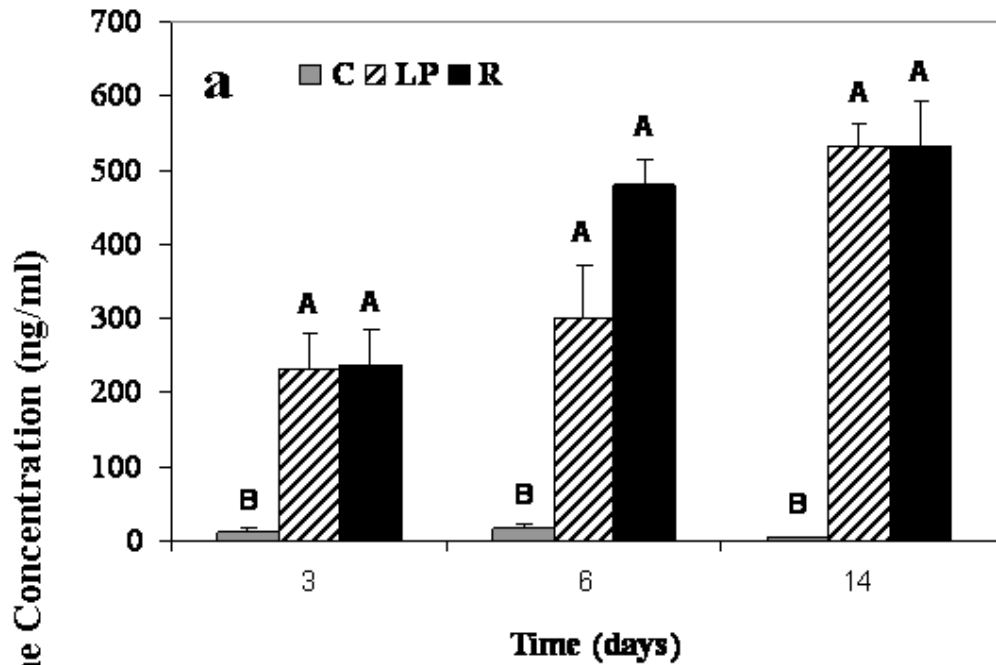
<sup>a</sup> Mean values, n=8 (4 males and 4 females) for each dietary group within each experimental period. Within a row, values not sharing a superscript letter differ ( $P \leq 0.05$ ) according to Tukey's Studentized Range test. SEM is the pooled SEM.

<sup>b</sup> C, age-matched control group consuming complete diet ad libitum; LP, group consuming low-protein diet ad libitum; R, group consuming complete diet in restricted daily quantities.

<sup>c</sup> cumulative intake for the experimental period

<sup>d</sup> From ANOVA of natural log-transformed data. Means are antilogs of log means.

<sup>e</sup> From ANOVA of square root-transformed data. Means are squares of square root means.



**Figure 1.** Concentrations of corticosterone in the serum of weanling C57BL/6J mice.

Male and female mice, initially 19 days old, were given free access to a complete purified diet (group C), or to a low-protein diet (group LP), or were fed the complete diet in restricted daily quantities (group R). The feeding period is indicated on the X-axis of each graph. Corticosterone levels were determined by (a) double antibody radioimmunoassay, or (b) competitive binding enzyme immunoassay. Bars represent mean values and are accompanied by SEM. Means are antilogs of log means in (a) days 3 and 14, and (b) day 3. Sample sizes were (a) 8 (3 days), 8 (6 days) and 11 (14 days) and (b) 5 (both feeding periods). Within each graph and feeding period, bars not sharing a lower-case letter differ ( $P \leq 0.05$ ) according to Tukey's Studentized Range test. (a) Pooled SEM = 0.37 (3 days), 71.46 (6 days) and 0.29 (14 days). (b) Pooled SEM = 0.53 (3 days) and 83.00 (14 days).

## **Discussion**

These experiments demonstrate that the blood total corticosterone concentration rises to levels associated with the highest illness severity scores (16) early in the response to acute weanling deficits of protein and energy. Importantly, the levels of serum glucocorticoids in the experimental systems used herein are similar to those reported in acutely malnourished children (2-6). This similarity underscores the relevance of appropriately crafted murine models of acute protein and energy deficit as discussed elsewhere (17) and adds to previous evidence (e.g., 13) specifically demonstrating the relevance to diverse forms of acute pediatric malnutrition on the part of the weanling models used herein. It is also noteworthy that the outcome of this investigation was apparent in genetically distant murine strains (10) and was seen independently of infection. In addition, these results were independent of both gender and the immunochemical technique applied to the hormone assays, including the increasingly popular competitive binding immunoassay procedure. For several reasons collectively, therefore, confidence is warranted in the broad applicability of these results.

The main contribution of the present investigation is to reveal the size of the glucocorticoid response to acute malnutrition which is at least an order of magnitude greater than previously recognized (e.g., 2-8). This outcome was made possible by application of an improved exsanguination procedure (9) that reduces acute pre-anesthesia stress, thereby permitting definition of the blood glucocorticoid concentration of the quiescent animal. Blood levels of growth hormone have occasionally been reported to rise (6), and blood levels of insulin to fall (6), by an order of magnitude in pediatric kwashiorkor and marasmus. Thus, the glucocorticoids must be added to a select

shortlist of endocrine hormones for which such large changes in blood concentration are reported in connection with the attempt to adapt to acute malnutrition. In addition, these results indicate that, together with a decrease in leptin levels that may permissively mediate the glucocorticoid response (1), an order-of-magnitude rise in blood glucocorticoid level is part of the early endocrinological response to acute deficits of protein and energy. The glucocorticoids directly regulate as many as 100 genes (18) that collectively promote mobilization of both lean and fat tissue (19) in support of vital metabolic functions and that exert other influences including potent anti-inflammatory immune suppression (1, 8, 18). In this connection, the early influx of glucocorticoids into the blood coincides with the shift toward catabolic metabolism (10, 11, this investigation) and the decline in cellular immune competence (10, 11) in the two experimental systems used herein. Therefore, the outcome of this investigation provides a new perspective on the significance of the glucocorticoid response in acute pediatric protein and energy deficit.

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## Chapter 5

**The blood level of transforming growth factor-beta rises in the early stages of acute protein and energy deficit in the weanling mouse.**

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## **Abstract**

Plasma transforming growth factor(TGF)- $\beta$  levels are high in the advanced stages of acute (wasting) pre-pubescent deficits of protein and energy. Consequently, this potently anti-inflammatory cytokine may help to sustain the depression of inflammatory immune competence in acute malnutrition. Our objective was to determine if plasma TGF- $\beta$  levels rise during the early stages of acute malnutrition and, secondarily, to confirm the elevation reported previously in advanced weight loss. In two experiments, male and female C57BL/6J mice, initially 19 days old, consumed a complete purified diet ad libitum (group C), or in restricted daily quantities (group R), or had free access to an isoenergetic low-protein diet (group LP). TGF- $\beta$  bioactivity in platelet-poor plasma was determined via inhibition of MV-1-Lu mink lung cell proliferation after 3 days (Experiment 1, early stage) or 14 days (Experiment 2, advanced stage) of dietary intervention. At 3 days, mean plasma TGF- $\beta$  bioactivities were 802 (C), 2952 (R) and 4678 (LP) pg/mL, and after 14 days mean bioactivities were 1786 (C), 5360 (R) and 5735 (LP) pg/mL. At both time points, the malnourished groups differed from age-matched controls ( $P \leq 0.05$ ). Thus, metabolically distinct weanling systems mimicking paediatric marasmus (group R) and kwashiorkor (group LP) exhibit an early rise in blood TGF- $\beta$  concentration, and this cytokine joins corticosterone and interleukin-10 as a third anti-inflammatory hormone temporally positioned to contribute to the initiation (and maintenance) of malnutrition-associated immune depression. This investigation contributes new insight into the actively anti-inflammatory form of immune competence that appears to prevail in acute pre-pubescent malnutrition.

## Introduction

A defining characteristic of acute (i.e. wasting) pre-pubescent protein and energy deficit is depressed inflammatory immune competence, although the etiology of this aspect of malnutrition pathology is poorly understood<sup>(1)</sup>. A recent proposition suggests that this immunological phenomenon represents a regulated pathophysiology, controlled by hormones and cytokines, rather than a biologically trivial disintegrative loss of immunological control<sup>(1)</sup>. In this connection, murine models that closely mimic acute paediatric malnutrition exhibit elevations in blood levels of three potent anti-inflammatory mediators, namely corticosterone<sup>(2)</sup>, interleukin(IL)-10<sup>(3)</sup> and transforming growth factor(TGF)- $\beta$ <sup>(3)</sup>, in the most advanced stages of wasting pathology. Moreover, in the same pre-pubescent experimental systems blood levels of both corticosterone<sup>(2)</sup> and IL-10<sup>(4)</sup> rise in the early stages of weight loss. Importantly, high blood levels of TGF- $\beta$  are also reported in the advanced stages of a completely different model of acutely protein deficient weanling guinea pigs vaccinated with *M. tuberculosis*<sup>(5)</sup>. Blood concentrations of hormones and cytokines represent spillover from the extravascular compartment and may be regarded as reflective, although probably not representative, of concentrations at extravascular sites of action<sup>(3,6)</sup>. High levels of glucocorticoids and IL-10, therefore, are positioned temporally to initiate depressed inflammatory capacity in the early stages of acute malnutrition, and a potent triad including the glucocorticoids, IL-10 and TGF- $\beta$  is likewise positioned to serve in a sustaining role. It is unknown whether TGF- $\beta$  might also contribute to the initiation of malnutrition-associated depression in inflammatory immune competence.

TGF- $\beta$  exerts potently anti-inflammatory and immune suppressive effects impacting cells of both the innate and adaptive arms of immune defence<sup>(7)</sup>, and this cytokine is regarded as a dominant peripheral mediator of anti-inflammatory self-tolerance<sup>(8,9)</sup>. The objective of this investigation was to determine if the blood TGF- $\beta$  concentration rises during the early stages of acute pre-pubescent malnutrition in metabolically distinct murine models known to depress inflammatory immune competence<sup>(10-12)</sup>. Additionally, a confirmatory experiment was conducted assessing blood levels of TGF- $\beta$  in the advanced stages of acute weanling malnutrition.

## **Materials and Methods**

### *Animals and facilities*

Male and female C57BL/6J mice were obtained from an in-house breeding colony. Caging and housing conditions were exactly as described previously<sup>(2, 12)</sup>, and this investigation was approved by the Animal Care Committee of the University of Guelph in accordance with the guidelines of the Canadian Council on Animal Care.

### *Diets and feeding protocols*

At 18 days of age the animals were weaned, individually housed and given free access for 1 day to a complete purified diet which is described elsewhere<sup>(13)</sup>. At 19 days of age, the mice were randomly allocated to one of three experimental groups. An age-matched control group was given ad libitum access to the aforementioned complete purified diet. An energy-restricted group consumed the complete diet in restricted daily quantities determined by calculations that relate ad libitum food intake (g food/g body

weight) to chronological age in the weanling mouse<sup>(12)</sup>. Finally, a low-protein group was given free access to a purified diet formulated to contain 0.6% crude protein by replacement of most of the nitrogen source of the complete diet by cornstarch<sup>(10)</sup>. These malnutrition protocols reproduce the critical features of the paediatric human pathologies of marasmus via the restricted-intake protocol and incipient kwashiorkor via the low-protein protocol<sup>(2, 3, 12)</sup>.

### *Experimental Design*

Two experiments were performed. In the first experiment, 10 animals were included in each dietary group and blood samples were taken at day 3, representative of the early stages of weight loss<sup>(2)</sup>. In the second experiment, intended to be confirmatory of a previous report pertaining to the advanced stages of malnutrition<sup>(3)</sup>, sample sizes consisted of 10 animals in the age-matched control group and 8 animals in both malnourished groups, and blood was taken after 14 days. Equal numbers of males and females were included in all dietary groups from both experiments.

### *Blood Sampling Procedure*

Orbital plexus blood samples were collected under CO<sub>2</sub> anaesthesia as described previously<sup>(3)</sup>. Platelet-poor plasma was collected and stored at -80°C as described previously<sup>(3)</sup>.

### *Plasma TGF- $\beta$ bioassay*

The assay was based on the reduction of the proliferative activity of MV-1-Lu mink lung cells (ATCC CCL-64) exposed to TGF- $\beta$  and was performed as described in

detail elsewhere<sup>(3)</sup>. Recombinant human TGF- $\beta$ 1 (BD Biosciences, catalog #559119) was used to generate standard curves (average  $R^2=0.98$ ), and only linear portions of the curves were used. The intra-assay coefficient of variation averaged 4.6% and the detection limit was 46 pg/mL. Each plasma sample was analyzed in triplicate.

### *Carcass Composition*

Carcasses were stored at -80° C to await analysis of dry matter and total lipid content as described previously<sup>(10)</sup>.

### *Statistical Analysis*

Statistical analyses were conducted using the SAS system (SAS Institute, Cary, NC) for Windows (version 9.0), and a pre-determined upper limit of probability of  $P \leq 0.05$  was applied for statistical significance. Data were subjected to a one-way ANOVA followed, if justified by Tukey's Studentized Range test. Data sets not exhibiting a normal distribution were transformed. Where transformation attempts were unsuccessful, data were subjected to the Kruskal-Wallis test ( $\chi^2$  approximation) followed, if justified by Wilcoxon two-sample testing.

## **Results**

### *Distinct weight loss pathologies were elicited by the malnutrition protocols*

Growth indices for the two experiments are shown in Table 1. Within each experiment, initial body weights did not differ among groups. Moreover, the age-matched control groups exhibited food intakes and carcass composition outcomes

comparable to those reported previously for C57BL/6J weanlings consuming the complete purified diet<sup>(2, 3, 12)</sup>. At the end of each experiment, the body weights and food intakes of the malnourished groups were lower than their corresponding age-matched control group. Also, each malnourished group exhibited a lower carcass fat level than its corresponding age-matched control, and the restricted-intake protocol induced a greater decrement in carcass fat, and hence in carcass energy, than the low-protein protocol. Overall, the loss of both fat and lean tissue produced by the two malnutrition protocols was comparable to the outcomes reported previously when inflammatory immune competence was shown to be depressed in the same experimental systems<sup>(10-12)</sup>.

#### *Plasma TGF- $\beta$ Bioactivity*

Plasma TGF- $\beta$  bioactivities are shown in Figure 1. Samples from all groups exhibited plasma bioactivities that exceeded the detection limit of the assay. Plasma TGF- $\beta$  bioactivities found in the age-matched control groups were consistent with concentrations reported previously in plasma samples from healthy adolescent and young adult mice<sup>(3, 14)</sup>. In relation to the main objective of this investigation, plasma TGF- $\beta$  bioactivities were elevated in both malnourished groups after only 3 days of weight loss and the high blood levels of this cytokine were sustained into the advanced stages of protein and energy deficit.

Table 1. Initial and Final Body Weights, Food Intakes, and Carcass Compositions<sup>a</sup>

Index	Dietary Group <sup>b</sup>			SEM
	C	LP	R	
	Day 3			
Initial body weight (g/mouse)	7.9	8.2	8.2	0.09
Final body weight (g/mouse)	9.6 <sup>X</sup>	7.4 <sup>Y</sup>	6.9 <sup>Y</sup>	0.09
Food intake (g/mouse · 3d)	2.2 <sup>X</sup>	1.6 <sup>Y</sup>	1.1 <sup>Z</sup>	0.03
Food intake (g/g body weight · day) <sup>c</sup>	0.17 <sup>X</sup>	0.13 <sup>Y</sup>	0.1 <sup>Z</sup>	0.001
Carcass dry matter (g/100g wet weight) <sup>d</sup>	29.5 <sup>X</sup>	29 <sup>X</sup>	27.8 <sup>Y</sup>	----
Carcass lipid (g/100g wet weight)	8.9 <sup>X</sup>	6.6 <sup>Y</sup>	3.9 <sup>Z</sup>	0.14
	Day 14			
Initial body weight (g/mouse) <sup>e</sup>	8.4	8.5	8.4	0.006
Final body weight (g/mouse) <sup>f</sup>	18.3 <sup>X</sup>	6.2 <sup>Y</sup>	6.4 <sup>Y</sup>	0.01
Food intake (g/mouse · 3d) <sup>g</sup>	2.6 <sup>X</sup>	1.2 <sup>Y</sup>	0.9 <sup>Z</sup>	----
Food intake (g/g body weight · day) <sup>f</sup>	0.15 <sup>X</sup>	0.1 <sup>Y</sup>	0.07 <sup>Z</sup>	0.03
Carcass dry matter (g/100g wet weight) <sup>h</sup>	32.0 <sup>X</sup>	29.1 <sup>Y</sup>	27.8 <sup>Y</sup>	----
Carcass lipid (g/100g wet weight)	9.6 <sup>X</sup>	4.8 <sup>Y</sup>	2.2 <sup>Z</sup>	0.17

<sup>a</sup> Values are means, day 3: n=10/dietary group, day 14: n=10 for group C and n=8 for groups LP and R. Within rows, values not sharing a superscript letter differ ( $P \leq 0.05$ ) according to Duncan's New Multiple Range Test, unless a different statistical procedure is indicated.

<sup>b</sup> C: fed complete diet ad libitum; LP: fed ad libitum a low-protein diet; R: energy deficient group fed complete diet in restricted daily quantities.

<sup>c</sup> From ANOVA of squared-transformed data. Mean values are square roots of squared means.

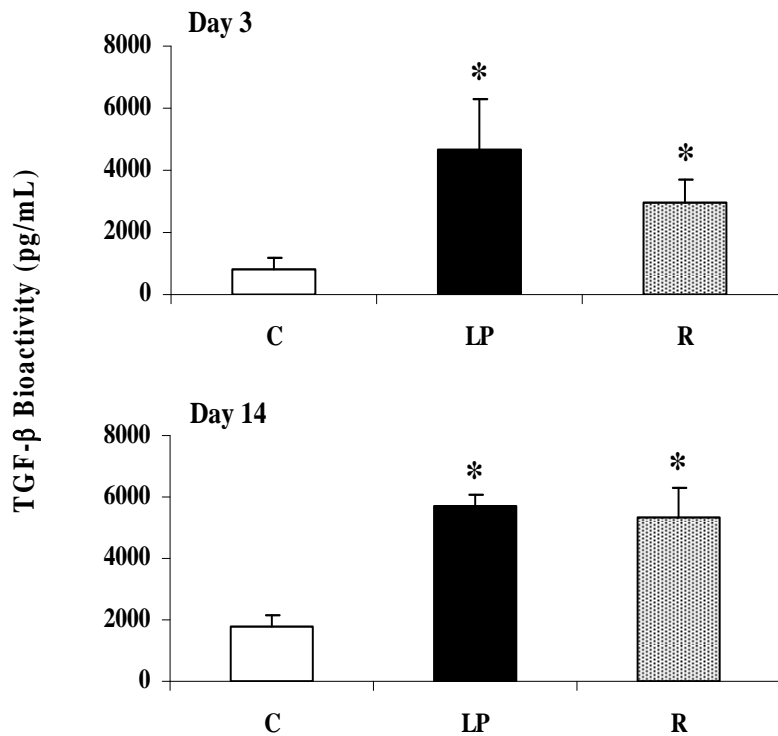
<sup>d</sup> Kruskal-Wallis test of Wilcoxon rank sums that were as follows: C, 187; LP, 188; R, 90.

<sup>e</sup> From ANOVA of inverse-transformed data. Mean values are the inverse of the transformed means.

<sup>f</sup> From ANOVA of natural log-transformed data. Mean values are antilogs of log means.

<sup>g</sup> Kruskal-Wallis test of Wilcoxon rank sums that were as follows: C, 215; LP, 98.5; R, 37.5.

<sup>h</sup> Kruskal-Wallis test of Wilcoxon rank sums that were as follows: C, 202; LP, 83.5; R, 65.5.



**Figure 1.** Plasma TGF- $\beta$  bioactivity. Weanling C57BL/6J mice, initially 19 days old, were fed a complete purified diet ad libitum (group C, age-matched controls), or the complete diet in restricted daily quantities (group R), or were given free access to an isoenergetic low-protein diet (group LP). Equal numbers of males and females contributed to samples sizes of 10 in all dietary groups (upper panel, day 3), or to sample sizes of 10 (group C) and 8 in each malnourished group (lower panel, day 14). Bars represent mean values and the SEM is shown in each case. Within each experiment, data were analyzed by one-way ANOVA. Day 3: Means are antilogs of natural log-transformed means; diet  $P=0.0004$ , pooled SEM=0.16. Day 14: diet  $P=0.0001$ , pooled SEM=339. Bars marked with an asterisk (\*) differ from their corresponding age-matched control group ( $P\leq 0.05$ ) according to Tukey's Studentized Range test.

## Discussion

This investigation reveals that the plasma concentration of TGF- $\beta$  rises early in response to acute pre-pubescent deficits of protein and energy, and demonstrates that the high levels of this anti-inflammatory cytokine are maintained into the advanced stages of weight loss. The findings pertaining to the later stages of malnutrition confirm previous reports<sup>(2, 5)</sup>. This outcome was apparent in two metabolically distinct murine models which mimic the critical features of paediatric marasmus and incipient kwashiorkor<sup>(2, 3, 12)</sup>. Thus, a potentially anti-inflammatory triad of soluble mediators, namely the glucocorticoids, IL-10 and TGF- $\beta$ , emerges when this work is considered together with previous reports<sup>(2-5)</sup> and provides a basis for understanding both initiation and maintenance of depressed inflammatory competence in acute pre-pubescent malnutrition. More broadly, this investigation provides new experimental underpinning for the concept<sup>(1)</sup> that malnutrition-associated immune depression is part of a regulated pathophysiology, the antithesis of a biologically trivial disintegration of immunological capacities.

Attempts to achieve a biologically meaningful assessment of TGF- $\beta$  concentrations in blood and other tissue fluids are complicated by the existence of three mammalian isoforms of this protein mediator, designated  $\beta$ 1-3,<sup>(15)</sup> and by the fact that much of the cytokine circulates in latent form<sup>(16)</sup>. Only the  $\beta$ 1 isoform is detectable in the blood of the healthy mouse or of mice subjected to the low-protein protocol used herein, whereas both  $\beta$ 1 and  $\beta$ 2 isomers are reported at high levels in the blood of mice subjected to our food intake restriction protocol<sup>(3)</sup>. It is important, therefore, that the three mammalian isoforms exhibit indistinguishable immunological influences, at least in

vitro,<sup>(3)</sup> and equal biological activity in the bioassay used herein<sup>(15)</sup>. Moreover, TGF- $\beta$  immunoassays<sup>(17)</sup> and bioassays<sup>(5, 17)</sup> frequently include an initial activation step that releases the cytokine from its latent form, thereby inflating the measurement that is interpreted as biologically active TGF- $\beta$ . However, this assay strategy was not used in the present investigation. To the extent that blood cytokine concentrations reflect extravascular levels<sup>(3, 6)</sup>, therefore, the TGF- $\beta$  concentrations determined herein are directly relevant to the levels of biologically active cytokine that target cells would encounter in vivo.

Numerous possibilities merit investigation as contributors to high circulating levels of TGF- $\beta$  in acute malnutrition. In this connection, findings pertaining to the small intestinal epithelium and lamina propria of acutely malnourished infants and children lend no support to the possibility of an increase in the rate of cytokine synthesis<sup>(18)</sup>. However, a much more extensive cellular survey is needed as virtually all cells can produce TGF- $\beta$ <sup>(16)</sup>. Likewise, reduced turnover must be considered in parallel with findings reported for other circulating proteins of acutely malnourished children, e.g. IgG class immunoglobulins<sup>(19)</sup> and some acute-phase proteins<sup>(20, 21)</sup>. Thirdly, an intriguing possibility pertains to the extracellular matrix which appears to serve as a reservoir for TGF- $\beta$ <sup>(16, 22)</sup>. Proteolytic degradation of the extracellular matrix liberates TGF- $\beta$  from its latent complex, thereby coupling matrix turnover with the activation of TGF- $\beta$ <sup>(22)</sup>. Thus, by exaggerating a normal physiological phenomenon, the catabolic metabolism of acute malnutrition may support a rapid and sustained elevation in blood and tissue fluid concentrations of a potent anti-inflammatory cytokine in its active form. In this

connection, protein and calorie deprived rats exhibit decreased soluble and insoluble collagen skin content in comparison to healthy controls<sup>(23)</sup>.

The glucocorticoids, IL-10 and TGF- $\beta$  comprise a network of partially redundant anti-inflammatory hormonal mediators effective against diverse innate and adaptive immune defence elements and widely regarded as the backbone of peripheral tolerance<sup>(7-9, 24, 25)</sup>. The present investigation completes the picture with respect to this potent network in acute pre-pubescent malnutrition. It is now apparent, from this investigation and other reports<sup>(2-4)</sup>, that the three component mediators rise early and are sustained in unison throughout the progression of weight loss. In turn, this points to the recent proposition of a coordinated and purposeful immune regulation in which the inflammatory form of competence is supplanted by an anti-inflammatory form that is tolerant of the self antigens catabolically released in quantity during wasting malnutrition<sup>(1, 3)</sup>. This tolerance-centered model emphasizes the adaptive benefit of a reduced risk of autoimmune disease while acknowledging the cost in terms of susceptibility to infectious disease. Other anti-inflammatory mediators, e.g. IL-35<sup>(24)</sup>, can be accommodated within the tolerance model and their participation can be anticipated in the non-inflammatory immune competence of acute pre-pubescent malnutrition.

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## Chapter 6

### **Elevated Blood Interleukin-10 Levels and Undiminished Systemic Interleukin-10 Production Rate Prevail Throughout Acute Protein-Energy Malnutrition in the Weanling Mouse**

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## **Abstract**

The objectives were to determine if blood IL-10 levels rise during the early stages of acute (wasting) pre-pubescent malnutrition in metabolically distinct murine models known to depress inflammatory immune competence and whether systemic IL-10 production is affected in these pathologies. Weanling C57BL/6J mice were assigned to dietary protocols that elicited wasting pathologies mimicking the human diseases of marasmus (restricted-intake group) or incipient kwashiorkor (low-protein group). An age-matched control group also was included. Serum IL-10 bioactivities were assessed in the early (day 3) and advanced (day 14) stages of weight loss, and net systemic IL-10 production was assessed at the same stages of pathology by *in vivo* cytokine capture. Blood IL-10 levels were elevated in both malnourished groups relative to controls at days 3 and 14 (range of P values: 0.03 – 0.0001). Further, despite a limited supply of energy and nitrogenous substrates, the systemic IL-10 production rate was at least sustained in the malnourished groups and, in fact, was elevated in the marasmic group (P=0.05) throughout the progression of weight loss. IL-10 emerges as an anti-inflammatory mediator positioned to participate in initiating and upholding the depressed immune competence that accompanies acute pre-pubescent deficits of protein and energy.

## 1. Introduction

Acute (i.e., wasting) pre-pubescent deficits of protein and/or energy in their most severe forms, i.e. marasmus and kwashiorkor, are associated with up to two million largely infection-related deaths annually among children under the age of five years, and this toll is undoubtedly exceeded by the additional burden of infection-related morbidity [1]. Depressed inflammatory immune competence is widely accepted as the link between malnutrition and susceptibility to opportunistic infection [2], and repairing the capacity to generate inflammations has been identified authoritatively as one of three preferred complementary approaches to reducing the burden of malnutrition-associated infection [3]. Rational, targeted immunological interventions, however, must await substantive improvement in mechanistic understanding of the physiological basis underlying malnutrition-associated immune depression [2].

A recent proposition suggests that immune depression in response to acute malnutrition represents a regulated pathophysiology, controlled by altered concentrations of hormones and cytokines, rather than a disintegrative loss of immunological control [4]. In this connection, appropriately crafted murine models exhibit profound elevations in blood levels of three anti-inflammatory and immune suppressive hormones, namely glucocorticoids [5], transforming growth factor(TGF)- $\beta$  and interleukin(IL)-10 [6], in the most advanced stages of acute weanling malnutrition. Clearly, this potent hormonal triad helps to sustain immune depression in the latter stages of wasting malnutrition. Moreover, a profound rise in glucocorticoid levels early in the progress of pre-pubescent malnutrition implicates this hormone in the initiation of immune depression [5], and it is of interest to pursue other members of the anti-inflammatory triad from the same point of

view. The present investigation centres on IL-10 which is a key regulator of both innate and adaptive immune responses [7,8]. Therefore, the first objective of this investigation was to determine if the blood IL-10 concentration rises during the early stages of acute pre-pubescent malnutrition in metabolically distinct murine models known to depress adaptive inflammatory cell-mediated immune competence [9-11]. The experimental strategy included a confirmatory assessment of blood IL-10 levels in the advanced stages of the same weight loss pathologies.

A high blood level of IL-10 could reflect a high cytokine production rate, a low rate of cytokine removal including catabolism, or both. In this connection, intracellular cytokine staining revealed a higher percentage of IL-10-producing T cells in the blood of children with various degrees and forms of acute malnutrition compared to well-nourished children [12,13]. A similar outcome is reported in relation to expression of IL-10 mRNA by freshly isolated blood mononuclear cells of acutely malnourished children [14]. These findings point to the possibility that acute pediatric malnutrition may promote an increase in IL-10 production despite obvious limitations in the availability of amino acids and energy. Blood T cells, however, cannot be regarded as representative of T cells systemically [15]. Moreover, diverse and anatomically disperse types of cells produce IL-10 in addition to T cells [7,8,16]. Since blood cytokines represent spillover from their numerous extravascular sources [17,18], use of the in vivo cytokine capture assay provides an index that relates to the net rate of systemic cytokine production independently of cellular source [19-21]. In brief, a biotin-conjugated IgG class anticytokine (“capture”) antibody binds to its target cytokine thereby extending the half-life of newly-synthesized cytokine molecules by forming biologically stable immune

complexes that accumulate in the blood [19-22]. The capture assay has been validated for several murine cytokines including IL-2, -4, -6, -10, interferon- $\gamma$  and tumor necrosis factor- $\alpha$  [20]. Therefore the second objective of this investigation was to use the in vivo cytokine capture assay to determine the influence of acute pre-pubescent deficits of protein and/or energy on an index relating to systemic IL-10 production at both the early and advanced stages of disease.

## **2. Materials and Methods**

### **2.1. Experimental Animals and Dietary Protocols**

C57BL/6J mice were obtained from an in-house breeding colony derived from animals originally purchased from the Jackson Laboratory (Bar Harbor, ME). At 18 days of age animals were weaned and acclimated to a complete purified diet for 1 day as described previously [21,23,24]. At 19 days of age, equal numbers of male and female mice were each randomly assigned to one of three experimental groups, namely an age-matched control group consuming a complete purified diet ad libitum as described in detail [23], an energy-restricted group consuming the complete purified diet in restricted daily quantities determined on the basis of calculations that relate ad libitum food intake (g food/ g body weight) to chronological age in the weanling mouse [11], and a low-protein group given free access to a 0.6% protein purified diet that is isocaloric with the complete formulation [6,18]. These long-established protocols of acute malnutrition elicit weight loss between 1.5 and 2 % of initial body weight daily throughout the experimental period [5,18,25], and the restricted-intake and low-protein protocols of dietary imbalance reproduce the critical features of the pre-pubescent human pathologies

of marasmus and incipient kwashiorkor respectively [5,6,11,18,25,26]. This investigation was approved by the Animal Care Committee of the University of Guelph in accordance with the requirements of the Canadian Council on Animal Care.

## 2.2. Experimental Design

Two experiments were performed, the first centered on blood IL-10 bioactivities and the second on application of the in vivo IL-10 capture assay. Each experiment was performed according to a 2 X 2 design (main effects: diet and time, i.e. stage of weight loss) and, in both experiments, blood samples were collected at 3 and 14 days of dietary intervention. At each time point assessed, equal numbers of males and females comprised sample sizes of 10 animals per dietary group (bioassay) and 8 animals per dietary group (in vivo capture assay). In addition, at each time point assessed (3 days and 14 days), four negative control mice (2 males and 2 females) were included with each dietary group of eight animals subjected to the in vivo capture assay.

## 2.3. Blood Sampling Procedure

Blood samples were collected from the orbital plexus while the animals were under CO<sub>2</sub> anesthesia as described [5] and the mice were then killed by cervical dislocation without recovering consciousness. Blood was allowed to clot at room temperature for 30-45 minutes and the resulting serum was stored -80°C to await analysis.

#### 2.4. IL-10 Bioassay

The IL-10 bioassay was conducted as described previously [6,27] and is based on the ability of IL-10 to inhibit interferon- $\gamma$  production by concanavalin A-stimulated murine spleen cells in vitro [27]. Only the linear portions of standard curves were used, and the reliability (intra-assay coefficient of variation) and detection limit of each assay were estimated as described elsewhere [27]. In this investigation, the standard curves for the assay were linear (mean  $R^2=0.98$ ) over a concentration range up to 7.5 ng/mL. All serum samples exhibited IL-10 bioactivities exceeding the detection limit of the assay which averaged 0.43 ng/mL, and the coefficient of variability for the bioassay averaged 8.6 %.

#### 2.5. In Vivo IL-10 Capture Assay

An in vivo cytokine capture assay kit for murine IL-10 (BD Biosciences, Mississauga, Canada, catalogue # 558072) was utilized according to the manufacturer's directions. Briefly, at the end of each feeding period (either 3 days or 14 days) each animal received an intraperitoneal injection of 10  $\mu$ g of a biotin-conjugated monoclonal rat anti-mouse IL-10 (capture) antibody in 200  $\mu$ L of endotoxin-free physiological saline (Vétoquinol N.-A. Inc., Lavaltrie, Québec; henceforth, "saline"). Blood samples were collected 4 h later to permit analysis of the concentration of newly-secreted IL-10 that had accumulated in molecular complexes with the capture antibody. An IL-10 sandwich ELISA was used to detect the antibody/cytokine complexes and was performed with reagents provided in the capture assay kit (BD Biosciences). The capture reagent of the

ELISA was a rat monoclonal antibody that recognized a different IL-10 epitope than the biotin-conjugated antibody used in vivo, and the detection reagent was streptavidin conjugated with horse radish peroxidase. In a minor departure from the manufacturer's instructions, an additional blocking step was included using 10% fetal bovine serum (Sigma Chemical) in sterile phosphate-buffered saline (1.0 mM, pH 7.3) following the capture stage of the ELISA. Outcomes were quantified by optical density using a Vmax kinetic plate reader (Molecular Devices Corp., Menlo Park, CA) set for absorbance at 450 nm with wavelength correction based on absorbance at 570 nm. Within each dietary group, and at each time point assessed (3 days and 14 days), 2 males and 2 females were included as negative controls, receiving 200  $\mu$ L of saline without capture antibody by intraperitoneal injection.

In this investigation, the standard curves for the assay were linear (mean  $R^2=0.99$ ) over a concentration range up to 2000 pg/mL and only the linear portions of the curves were used. In addition, the reliability (intra-assay coefficient of variation) and detection limit of each assay were estimated as described previously [27]. All serum samples exceeded the limit of detection of the assay which averaged 0.14 pg/mL, and the coefficient of variability averaged 4.3 %.

## 2.6. Carcass Composition

Carcasses were stored at  $-80^{\circ}$  C to await analysis of dry matter and total lipid contents as described previously [5,11].

## 2.7. Statistical Analysis

Statistical analyses were conducted using the SAS system (SAS Institute, Cary, NC) for Windows (version 9.0), and a predetermined upper limit of probability of  $P \leq 0.05$  was applied for statistical significance. Data were subjected to a two-way ANOVA in which the main effects were diet and time (i.e. stage of weight loss, either day 3 or day 14 of dietary intervention). If justified by the level of statistical probability ( $P \leq 0.05$ ), main effects were subjected to Duncan's New Multiple Range test. Moreover, a statistically significant interaction term ( $P \leq 0.05$ ) permitted up to five (i.e., treatment degrees of freedom) pre-planned comparisons by least squares means analysis. These comparisons were between age-matched controls at day 3 and day 14 (to discern ontogeny) and between each malnourished group and the age-matched control at day 3 and at day 14. Interpretation of a significant interaction term was probed further by means of a Pearson correlation analysis. If a data set failed to exhibit normal distribution according to each of the four tests applied by the SAS program ( $P \leq 0.05$ ) then a transformation was utilized to bring it into conformity with this basic assumption of parametric testing. Where transformation attempts were unsuccessful, data were subjected to the Kruskal- Wallis test ( $\chi^2$  approximation) followed, if justified by the statistical probability outcome ( $P \leq 0.05$ ), by Wilcoxon two-sample testing.

## 3. Results

### 3.1. Distinct Weight Loss Pathologies Were Elicited by the Malnutrition Protocols

Growth indices are shown in Table 1 for the experiment centered on blood IL-10 bioactivities. The wasting disease produced by the two malnutrition protocols in their

early (3 days) and more advanced (14 days) stages was comparable to the outcomes reported previously when depressed thymus-dependent immune competence, including inflammatory cell-mediated responsiveness, was demonstrated in the same experimental systems [9-11,26,28,29]. Initial body weight did not differ among the dietary groups that were assessed at either time point, and the final body weights of the malnourished groups fell below the weight of their corresponding age-matched control group but did not differ from each other at either stage of weight loss assessed. The food intakes and carcass composition exhibited by the age-matched control groups were comparable to previous results pertaining to C57BL/6J weanlings given free access to the complete purified diet used in this investigation [5,6,11,18,24,25,30]. The malnourished groups exhibited decreased food intakes relative to their corresponding age-matched controls, as seen previously in both 3-day [5,11,25] and 14-day [5,11,18,24,25,30,31] protocols. The percentage of carcass fat was low in the restricted-intake group as early as day 3, whereas this was apparent in the low-protein group only in the more advanced stage of weight loss. Even in advanced wasting, the percentage of carcass fat continued to be lower in the restricted intake group than in the low-protein group. As discussed previously, therefore, the restricted intake protocol induces a greater decrement in carcass energy than the low-protein system [21,24]. Similar outcomes with respect to growth indices were apparent in relation to the experiment centered on assessment of systemic IL-10 production (results not shown).

### 3.2. Serum IL-10 Bioactivity

Two-way ANOVA revealed an effect of diet ( $P < 0.0001$ ), time ( $P = 0.004$ ) and an interaction term (diet x time,  $P = 0.03$ ), as represented in Figure 1. As shown previously [6] both malnourished groups, when in the advanced stages of weight loss (14 days), exhibited high serum IL-10 bioactivity in comparison to age-matched controls. More importantly, both malnourished groups also exhibited high serum IL-10 bioactivity relative to age-matched controls after only 3 days of malnutrition. A comparison between day 3 and day 14 age-matched controls revealed no influence of ontogeny (least squares means,  $P = 0.92$ ). The interaction term, therefore, reveals a rise in blood IL-10 concentration that begins early in the development of both forms of weanling malnutrition and is sustained into their advanced stages. Pearson correlation analysis facilitated interpretation of the interaction term. A significant positive correlation emerged between weight loss and serum IL-10 bioactivity in the restricted-intake group ( $r = 0.55$ ,  $P = 0.01$ ) but not in the low-protein group ( $r = 0.28$ ,  $P = 0.24$ ) when data from days 3 and 14 were combined. Thus, serum IL-10 level rose early in both forms of malnutrition but continued to rise throughout the progression of weight loss only in the restricted-intake (marasmic) model.

### 3.3. In vivo IL-10 capture

A two-way ANOVA revealed a diet main effect ( $P = 0.05$ ) but no effect of time ( $P = 0.86$ ) and no interaction term ( $P = 0.99$ ). Therefore, Figure 2 centers on the diet related main effect, i.e. by combining the data from days 3 and 14 for each dietary group

(n=16), and shows that the restricted intake group exhibited an elevated rate of IL-10 capture relative to age-matched controls through the four-hour period of the assay, whereas the low-protein group did not differ from age-matched controls. In the absence of a time-related main effect or an interaction term, this outcome reveals that the increase in the restricted-intake group was maximal at an early stage of weight loss (day 3) and that the low-protein group was able to sustain an unchanged rate of IL-10 capture within the vascular compartment into the advanced stages of pathology. The results shown in the figure incorporate findings pertaining to negative control animals which, according to a two-way ANOVA, exhibited a diet-related false positive signal ( $P < 0.0001$ ) that was independent of the stage of weight loss (interaction term,  $P = 0.59$ ). No main effect emerged among negative controls relating to the stage of wasting disease ( $P = 0.60$ ). Therefore, to produce the results shown in Figure 2, the data from each dietary group were corrected prior to statistical analysis by subtraction of the mean value representing the eight mice (four from day 3 and four from day 14) of the corresponding negative control group (age-matched controls, 128 pg/mL; low-protein, 33 pg/mL; restricted-intake, 7 pg/mL).

Table 1. Experiment 1 (Serum IL-10 Bioactivity): Initial and Final Body Weights, Food Intakes, and Carcass Compositions<sup>1</sup>

Index	Dietary Group <sup>2</sup>			SEM
	C	LP	R	
	Day 3			
Initial body weight (g/mouse)	8.3	8.4	8.4	0.05
Final body weight (g/mouse)	9.8 <sup>x</sup>	7.5 <sup>y</sup>	7.4 <sup>y</sup>	0.07
Food intake (g/mouse · 3 d)	2.3 <sup>x</sup>	1.3 <sup>y</sup>	1.1 <sup>z</sup>	0.04
Food intake (g/g body weight · d) <sup>3</sup>	0.17 <sup>x</sup>	0.11 <sup>y</sup>	0.09 <sup>z</sup>	0.02
Carcass dry matter (g/100g wet weight)	30.5 <sup>x</sup>	29.2 <sup>x</sup>	27.7 <sup>y</sup>	0.25
Carcass lipid (g/100g wet weight)	6.4 <sup>x</sup>	5.5 <sup>x</sup>	3.5 <sup>y</sup>	0.45
	Day 14			
Initial body weight (g/mouse) <sup>3</sup>	8.3	8.3	8.2	0.05
Final body weight (g/mouse) <sup>4</sup>	18.4 <sup>x</sup>	6.2 <sup>y</sup>	6.2 <sup>y</sup>	0.001
Food intake (g/mouse · 14 d) <sup>3</sup>	3.3 <sup>x</sup>	1.4 <sup>y</sup>	0.8 <sup>z</sup>	0.02
Food intake (g/g body weight · d) <sup>3</sup>	0.19 <sup>x</sup>	0.12 <sup>y</sup>	0.07 <sup>z</sup>	0.03
Carcass dry matter (g/100g wet weight) <sup>5</sup>	32.6 <sup>x</sup>	28.8 <sup>y</sup>	28.0 <sup>y</sup>	----
Carcass lipid (g/100g wet weight)	9.8 <sup>x</sup>	4.4 <sup>y</sup>	2.5 <sup>z</sup>	0.20

<sup>1</sup>Values are means. At each time point, n=10/dietary group. Within a row, values not sharing a superscript letter (x, y, z) differ (P≤0.05) according to Duncan's New Multiple Range test, unless a different statistical procedure is indicated. SEM: pooled standard error of the mean.

<sup>2</sup>C: group that consumed the complete diet ad libitum; LP: group that consumed an isocaloric low-protein diet ad libitum; R: energy-deficient group that consumed the complete diet in restricted daily quantities

<sup>3</sup>from ANOVA of natural log-transformed data. Mean values are antilogs of log means.

<sup>4</sup>from ANOVA of inverse-transformed data. Mean values are the inverse of the transformed means.

<sup>5</sup>Kruskal-Wallis test of Wilcoxon rank sums that were as follows: C, 233; LP, 142; R, 90.

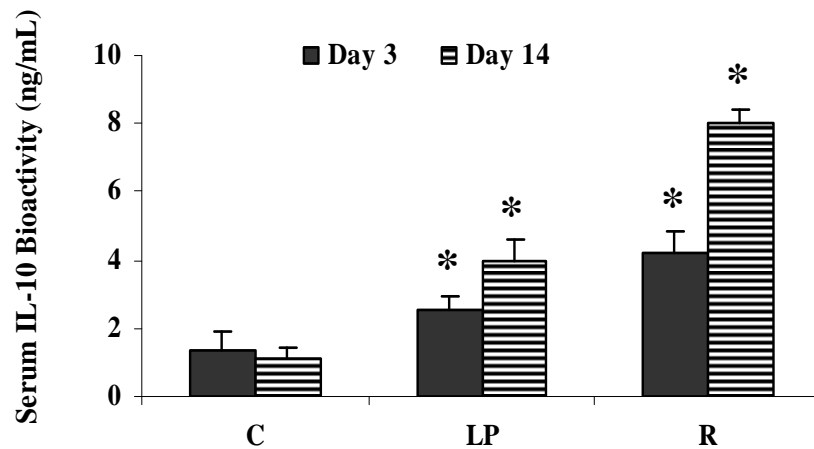


Figure 1. Serum IL-10 Bioactivity. Male and female weanling C57BL/6J mice, initially 19 days old, were fed a complete purified diet ad libitum (group C, age-matched controls), or the complete diet in restricted daily quantities (group R) or were given free access to an isocaloric low-protein diet containing 0.6% crude protein (group LP). Both malnutrition protocols elicited daily losses of approximately 1.5-2% of initial body weight. The feeding period was either 3 days (solid bars) or 14 days (striped bars). At each time point assessed, samples comprised 5 males and 5 females within each dietary group. Outcome of a two-way ANOVA, (main effects: diet and time, i.e. day 3 or 14): diet  $P < 0.0001$ , time  $P = 0.004$ , interaction  $P = 0.03$ , pooled SEM = 0.063. Bars represent squared means of square root transformed data and SD is shown. Within time points, bars marked with an asterisk (\*) represent a statistically significant difference from the

corresponding age-matched control group ( $P \leq 0.05$ ) according to least squares means analysis.

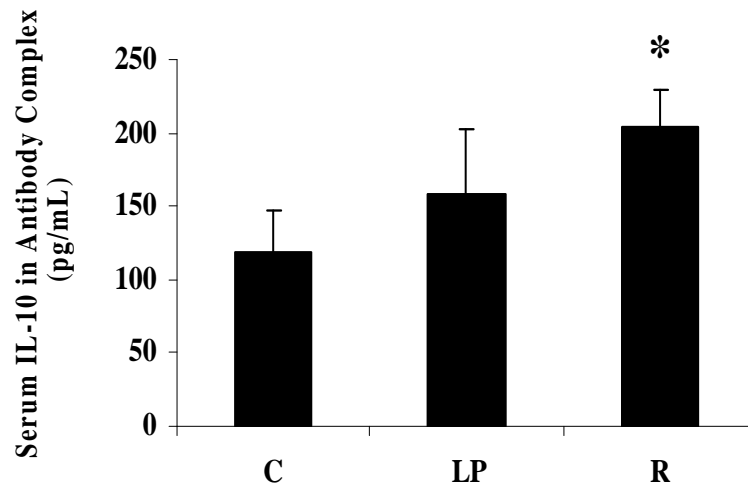


Figure 2. Serum concentration of IL-10 complexed with biotin-conjugated anti-IL-10 antibody from C57BL/6J mice four hours after intraperitoneal injection of 10  $\mu$ g of the biotin-tagged capture antibody. Mice initially 19 days old, were fed a complete purified diet ad libitum (group C, age-matched controls), or were given the complete diet in restricted daily quantities (group R), or were given free access to an isocaloric low-protein diet containing 0.6% crude protein (group LP). Both malnutrition protocols elicited daily losses of approximately 1.5-2% of initial body weight. Within each feeding period (3 or 14 days), each dietary group included 4 males and 4 females. A two-way ANOVA revealed a diet main effect ( $P=0.05$ ) but no main effect of time (i.e., stage of weight loss, 3- or 14-day feeding period,  $P=0.86$ ) and no interaction term ( $P=0.99$ ).

Pooled SEM=0.05. Hence, the graph shows only the diet effect outcome in which data within each dietary group are combined across time periods (n=16). Bars represent antilogs of means of natural log-transformed data and SD is shown. Within each dietary group, data were corrected before the statistical analysis by subtraction of the mean value of the corresponding negative control group (n=8, 4 males and 4 females). The asterisk (\*) represents a statistically significant difference ( $P \leq 0.05$ ) from the age-matched control group according to Duncan's New Multiple Range test.

#### **4. Discussion**

The present investigation revealed that the blood bioactivity of the anti-inflammatory cytokine, IL-10, is elevated during the early stages of acute pre-pubescent malnutrition. Moreover, a previous report [6] is confirmed that high blood IL-10 levels are maintained into the advanced stages of weight loss. In partial explanation of these findings, application of the in vivo cytokine capture assay revealed that systemic production of IL-10 was at least sustained in acute forms of malnutrition even as weight loss progressed into its most advanced stages. These experimental results were apparent in two metabolically distinct murine models of acute pre-pubescent malnutrition which consistently depress adaptive inflammatory immune competence [9-11] and which mimic the well-defined pediatric pathologies of marasmus via the restricted intake model and incipient kwashiorkor via the low-protein model [6,11,18,31,32]. In fact, systemic IL-10 production was increased by the marasmic form of malnutrition. By analogy with blood endocrine hormones [18], blood cytokines represent spillover from the extravascular compartment and their concentrations can be interpreted to reflect (but not to represent) cytokine levels at sites wherein immune responses arise [17,18]. Thus, IL-10 emerges as an anti-inflammatory mediator positioned to play a part in both initiating and sustaining malnutrition-associated immune depression. This conclusion is based, in substantial part, on the use of a bioassay which, unlike the popular sandwich enzyme-linked immunosorbent assay (ELISA), detects total biological activity rather than only a tiny and unrepresentative unbound biochemical fraction of unknown biological activity [17,27].

The metabolic dissimilarities between mice subjected to the low-protein and restricted-intake protocols, and their respective similarity to the human pathologies of kwashiorkor and marasmus have been noted elsewhere [24,31,32]. Briefly, the low-protein diet produces weight loss consequent to dietary imbalance, and weanling mice consuming this diet develop fatty liver and edema, the hallmark signs of kwashiorkor, after four weeks or more of wasting disease. When imposed for the periods of time reported in this investigation, therefore, the low-protein protocol produces an incipient kwashiorkor. In contrast, the restricted-intake protocol induces weight loss apart from dietary imbalance, and produces neither fatty liver nor edema, but stimulates the extreme mobilization of fat reserves that would be expected in a model of marasmus. By way of further contrast, the restricted-intake protocol produces a vigorously gluconeogenic animal whereas the low-protein diet, with its high level of digestible carbohydrate, has the opposite metabolic influence [32,33]. Nevertheless, these forms of experimental malnutrition exhibit immunological (including cytokine) characteristics that appear to differ only quantitatively but not qualitatively [4,6,18,21,24,26,31], and the findings reported herein appear consistent with this broadly based conclusion. In particular, a recent study showed that a distinction between the restricted-intake and low-protein models in the rate of systemic interferon- $\gamma$  production disappeared when the models were compared at similar levels of carcass energy decrement [21]. A dedicated search for biologically substantive immunological distinctions between, or among, forms of acute malnutrition is of much interest but is beyond the design and scope of this investigation.

No *in vivo* index of cytokine production can be independent of physiological turnover, a potentially crippling confounder in relation to blood cytokines because of

their brief half-lives that are generally measured in minutes [34-37]. The in vivo cytokine capture assay traps newly-synthesized cytokine in an antibody complex that extends the half-life of the bound cytokine to hours or days in the blood of the mouse [22,35,37-39]. Therefore, when performed with a suitably short assessment period, e.g. 4 h as in the present investigation, the assay is not overwhelmed by the factor of turnover. In fact, this assay strategy is simply an extension of the physiological phenomenon whereby the clearance of cytokines from the blood is retarded when these molecules form complexes with soluble cytokine receptors [40] or anti-cytokine autoantibodies [41]. Thus, the in vivo capture assay as applied in this investigation permits assessment of the rate at which a cytokine enters the vascular compartment, an index directly related to systemic production rate [19-21].

Malnutrition-associated increases in blood IL-10 levels can be attributed reasonably, at least in part, to a decrease in the rate of blood protein catabolism, a phenomenon already documented in acutely malnourished children with respect to IgG class immunoglobulins [42] and acute phase proteins including  $\alpha$ 1-antitrypsin and haptoglobin [43] and albumin [44]. However, the present investigation addresses the production component of the turnover equation showing that, despite limited intake of energy and nitrogenous substrates, systemic production of IL-10 was at least sustained in two metabolically distinct experimental systems even into the advanced stages of wasting pathology. Therefore, the high level of IL-10 that prevails in the blood, and undoubtedly in extravascular fluids, in these forms of weanling malnutrition is linked to a remarkable capacity to sustain production of IL-10 in the face of a profoundly limited supply of amino acids. Major producers of this cytokine include T and B cells, macrophages,

dendritic cells, mast cells, keratinocytes, adipocytes and fibroblasts [7,8,16,45] and it is of interest to determine which of these cellular elements is important to the maintenance of systemic IL-10 production in acute pre-pubescent malnutrition. In addition, it will be important to determine whether the inability of the model of incipient kwashiorkor to support an increase in systemic IL-10 production (by contrast with the model of marasmus) is a reflection of the particularly limited intake and availability of nitrogen in this model of dietary imbalance.

Interferon- $\gamma$  is a potent inflammatory cytokine counterbalanced in many respects by IL-10, e.g. in part via its influence on IL-12 [8,46-48]. It is noteworthy, therefore, that the models of weanling malnutrition used in this investigation exhibit a reduced blood bioactivity of interferon- $\gamma$  [18] and a reduced systemic production rate of this cytokine [21]. Considered in this context, the present investigation contributes new evidence consistent with the recent proposition that a physiologically regulated, anti-inflammatory (tolerance-centered) form of immune competence is sustained in acute malnutrition. This model represents the antithesis of the prevalent paradigm which propounds disintegrative loss of immunological control as the basis of reduced inflammatory capacity in acute malnutrition [2,4,6]. The tolerance model acknowledges the metabolic cost of physiological regulation as well as the subsequent risk of infection consequent to immune depression, but assigns priority to the immediate risk of inflammatory autoimmune reactions in the face of an overwhelming load of catabolically released self-antigens.

The advanced stages of acute weanling protein and energy deficit exhibit a remarkable elevation in blood levels of a triad of anti-inflammatory hormones, namely the glucocorticoids, TGF- $\beta$ 1 and IL-10 [5,6]. Moreover, the elevation in blood

glucocorticoid levels begins in the early stages of weight loss in the forms of experimental malnutrition pursued in the present investigation [5]. The findings reported herein, therefore, identify IL-10 as a second anti-inflammatory hormone positioned both to initiate immune depression in response to pre-pubescent deficits of protein and/or energy and to sustain this immunological influence into the advanced stages of wasting pathology.

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## Chapter 7

**IL-10 production declines in the innate anti-inflammatory response to lipopolysaccharide challenge as acute malnutrition progresses in the weanling mouse**

## **Abstract**

Constitutive interleukin(IL)-10 production represents a regulated anti-inflammatory form of immune competence sustained into the advanced stages of acute (wasting) pre-pubescent protein and energy deficit. In this investigation a second tier of anti-inflammatory defence, the innate response to lipopolysaccharide (LPS), was assessed in acute weanling malnutrition. Male and female C57BL/6J mice, initially 19 days old, consumed for up to 14 days a complete purified diet ad libitum (age-matched controls), an isocaloric low-protein diet ad libitum (mimicking incipient kwashiorkor), or the complete diet in restricted daily quantities (mimicking marasmus). In Experiment 1, IL-10 production by splenic and nodal mononuclear cells in response to LPS in vitro (10  $\mu\text{g}/\text{mL}$ , 24 hours) was unaffected by 14 days of acute malnutrition ( $P=0.47$ ). In Experiment 2, systemic innate-type IL-10 production during four hours following LPS challenge (1  $\mu\text{g}/\text{g}$  body weight) was assessed using the in vivo cytokine capture assay. Relative to controls, systemic innate-type IL-10 production in response to LPS in vivo was sustained in both malnourished groups in early-stage weight loss (day 3,  $P=0.19$  and  $0.46$ ) but was low in both malnourished groups at an advanced stage (day 14,  $P<0.0001$  and  $P=0.006$ ). Malnutrition-associated decline in second-tier innate-type IL-10 production is attributable to lymphoid involution and may constitute an early sign that physiological control over non-inflammatory immune competence is beginning to disintegrate.

**Keywords:** anti-inflammatory cytokine, interleukin-10, lipopolysaccharide, mouse, protein-energy malnutrition

## **Introduction**

Marasmus and kwashiorkor, the most severe forms of acute (i.e., wasting) protein-energy malnutrition (PEM), are associated with up to two million largely infection-related deaths annually in children under five years of age [1]. When infection-related morbidity is also considered, the numbers of children affected by these dietary pathologies undoubtedly exceed this estimate. Depressed inflammatory cell-mediated immune competence is widely accepted as a critical link between acute pediatric malnutrition and susceptibility to opportunistic infection [2,3] and is apparent also in animal models including murine systems which mimic the critical features of pediatric marasmus and kwashiorkor [4-6].

The hormonal triad of glucocorticoids, transforming growth factor(TGF)- $\beta$  and interleukin(IL)-10 is central to physiological anti-inflammatory control and the maintenance of self tolerance [7-12]. Thus, the constitutive blood levels of these mediators collectively represent the primary or first tier of immune regulatory competence which is in place, effectively in a sentinel role, when an inflammatory stimulus is first encountered. Recently, high constitutive blood levels of each member of this anti-inflammatory triad, namely corticosterone [13], TGF- $\beta$  [14,15] and IL-10 [14,16], have been reported in weanling mouse models of marasmus and incipient kwashiorkor. Further, remarkably, the systemic rate of constitutive IL-10 production is at least sustained even into advanced stages of wasting pathology [16]. These findings have given rise to the proposition that malnutrition-associated depression in inflammatory

capacities results from an up-regulation of tolerance-centered competence in the face of a large catabolic release of self antigens [14-16].

In addition to their constitutive production, anti-inflammatory mediators are a component of any competent response to inflammatory stimuli [17]. Such stimulus-dependent production may be regarded as a second tier of anti-inflammatory and tolerogenic immune regulation, and both innate and adaptive types of second-tier responses can be identified. IL-10 is of particular interest as a key regulator of both innate and adaptive responses [18,19]. The objective of this investigation was to assess, in vitro and in vivo, the influence of metabolically distinct forms of acute malnutrition on innate-type second tier production of IL-10 in weanling mouse models known to sustain constitutive production of this anti-inflammatory cytokine. Lipopolysaccharide (LPS), which potently activates innate immune defences through the toll-like receptor(TLR)-4 [20], was selected to elicit the experimental inflammatory stimulus.

## **Materials and Methods**

### ***Animals and Housing Facilities***

Male and female C57BL/6J mice were obtained from an in-house breeding colony, derived from animals originally purchased from the Jackson Laboratory (Bar Harbor, ME). Caging and housing conditions were exactly as described previously [4,6,21], and this investigation was approved by the Animal Care Committee of the University of Guelph in accordance with the Canadian Council on Animal Care.

## Diets and Experimental Design

Eighteen-day-old animals were weaned and acclimated overnight to a complete purified diet described in detail elsewhere [22]. Afterward, equal numbers of male and female 19-day-old mice were randomly assigned to one of three experimental groups, namely an age-matched control group consuming the complete purified diet ad libitum, a food intake-restricted group consuming the complete purified diet in restricted daily quantities based on calculations that relate ad libitum food intake (g food/g body weight) to chronological age in the weanling mouse [6], and a low-protein group consuming ad libitum a 0.6% crude protein diet that is isocaloric with the complete diet [14]. The restricted-intake protocol produces a pathology that mimics the critical features of pediatric marasmus, whereas the low-protein model closely resembles incipient pediatric kwashiorkor [6,13,14,23-25].

The capacity to respond to inflammatory stimulation via LPS was assessed both in vitro and in vivo. Two experiments were performed in which the three dietary groups were maintained on their respective regimens for 14 days, i.e. from 19 through 33 days of age. Additionally, in the second experiment, a cohort of animals was examined after only 3 days (i.e., from 19 through 22 days of age), a time point taken, as in previous studies [6,13], to represent the early stages of weight loss.

In the first experiment, a sample size of six per dietary group was used, and equal numbers of males and females were included in each of the experimental groups. In order to obtain sufficient numbers of cells (spleen combined with mesenteric and inguinal lymph nodes), pooled samples were required for mice subjected to the low-protein and restricted-intake groups. Each pooled sample (two mice for the low-protein group and

four mice for the restricted-intake group) included equal numbers of males and females and constituted a single degree of freedom for the purpose of statistical analysis. The experiment was conducted according to a 2 X 2 design in which diet and in vitro stimulation by LPS were the main effects, and cells were harvested for study at the end of the 14-day experimental period. In the second experiment, in vivo cytokine capture of IL-10 produced in response to an injection of LPS was assessed using blood samples taken at day 3 (early) and day 14 (advanced weight loss), thus yielding a 2 X 2 design in which the main effects were diet and stage of weight loss. In the LPS stimulated group, eight mice (four males and four females) were included within each dietary group at each time point, and pooling was unnecessary. Additionally within each dietary group and time point, unstimulated controls were included (n=16, eight males and eight females). Carcasses from animals of both experiments were stored at -20°C to await analysis.

### **Procedure to obtain mononuclear cell suspensions for in vitro stimulation**

After measurement of body weight, mice were killed with an overdose of CO<sub>2</sub> followed by cervical dislocation. The spleen, mesenteric and inguinal lymph nodes were removed aseptically, diced together and forced through a sterile stainless steel screen (100-mesh) into RPMI 1640 medium (Flow Laboratories, Mississauga, Canada) containing 10% heat-inactivated fetal bovine serum (Sigma Chemical, St. Louis, MO), 1 mmol/L HEPES (ICN Biomedicals, Aurora, OH), 10<sup>5</sup> U/L penicillin and 100 mg/L streptomycin (hereafter designated “complete medium”). A single-cell suspension of mononuclear cells was produced by discontinuous gradient centrifugation as described

previously [21]. Cell numbers were determined using a hemocytometer and viability, assessed by eosin Y exclusion, always exceeded 95%.

### **In vitro stimulation of mononuclear cells to elicit IL-10 production**

Each well of a 96-well V-bottom plate (catalog #249662, Nalge Nunc International), received  $2 \times 10^5$  viable mononuclear cells in 190  $\mu\text{L}$  complete medium. Subsequently, 10  $\mu\text{L}$  of complete medium was added to half of the culture wells to generate unstimulated negative control cultures while the remaining wells received 10  $\mu\text{L}$  of LPS (*Escherichia coli* 055:B5, Sigma–Aldrich, St. Louis, USA) diluted in complete medium to achieve a final concentration of 10  $\mu\text{g}/\text{mL}$  in each well. All cultures were run in triplicate and incubated at 37°C for 24 h. After incubation, supernatants from each set of triplicate wells were pooled, subdivided into aliquots and stored at -80°C.

### **Assay of IL-10 concentrations generated in vitro**

A sandwich ELISA kit for assay of mouse IL-10 (BD Biosciences, Mississauga, ON, catalogue #555252) was applied to samples as described by the manufacturer. Outcomes, run in triplicate, were quantified by optical density using a Vmax kinetic plate reader (Molecular Devices Corp., Menlo Park, CA) set for absorbance at 450 nm with wavelength correction based on absorbance at 570 nm. Only the linear portions of standard curves were used (mean  $R^2=0.97$ ) and all samples fell within this part of the curve. The reliability of the assay (intra-assay coefficient of variation) averaged 5.8% and its detection limit, determined as described elsewhere [26], averaged 2.2 pg/mL.

### **In vivo IL-10 capture assay**

An in vivo cytokine capture assay kit for murine IL-10 (BD Biosciences, Mississauga, Canada, catalogue # 558072) was utilized as described previously [16]. Briefly, each animal received an intraperitoneal injection of 10 µg of biotin-conjugated anti-mouse IL-10 (“capture”) antibody delivered in 200 µL of endotoxin-free physiological saline (Vétoquinol N.-A. Inc., Lavaltrie, Québec; henceforth, “saline”) plus 1 µg/gram body weight of LPS (*Escherichia coli* 055:B5, Sigma –Aldrich, St. Louis, USA). Unstimulated control animals received an injection of 10 µg of capture antibody alone in a 200 µL volume of saline. Blood samples were collected 4 h later to permit analysis of the serum concentration that had accumulated of molecular complexes between newly-secreted IL-10 and the capture antibody. A sandwich ELISA was used to detect the antibody/cytokine complexes and was performed with reagents provided in the capture assay kit (BD Biosciences). The capture reagent of the ELISA was a rat monoclonal antibody that recognized a different IL-10 epitope than the biotin-conjugated antibody used in vivo, and the detection reagent was streptavidin-conjugated horseradish peroxidase. In a minor departure from the manufacturer’s instructions, an additional blocking step was included using 10% fetal bovine serum (Sigma Chemical) in sterile phosphate-buffered saline (1.0 mM, pH 7.3) following the capture stage of the ELISA. Outcomes were quantified by optical density using a Vmax kinetic plate reader (Molecular Devices Corp., Menlo Park, CA) set for absorbance at 450 nm with wavelength correction based on absorbance at 570 nm.

The reliability (intra-assay coefficient of variation) and detection limit of each assay were estimated as described previously [26] and averaged 4.3% and 0.14 pg/mL (of

IL-10 in molecular complex) respectively. The standard curves for the assay were linear (mean  $R^2=0.99$ ) over a concentration range up to 2000 pg/mL. All samples fell within the linear portion of the curve and exceeded the detection limit of the assay.

### **Blood collection procedure for the in vivo capture assay**

As described previously [13] blood samples were collected from the orbital plexus while the mice were under CO<sub>2</sub> anesthesia, and the animals were subsequently killed by cervical dislocation without recovering consciousness. Samples were allowed to clot at room temperature for 30-45 minutes and the resulting serum was stored at -80°C to await analysis.

### **Carcass composition**

Analyses of dry matter and total lipid content were performed as described previously [6].

### **Statistical analysis**

Statistical analyses were conducted using the SAS system for Windows version 9.0 (SAS Institute, Cary, NC), and a predetermined upper limit of probability of  $P \leq 0.05$  was applied for statistical significance. Data were subjected to ANOVA followed, if justified by the level of statistical probability ( $P \leq 0.05$ ), by Tukey's Studentized Range test. If a data set failed to exhibit normal distribution according to each of the four tests applied by the SAS program ( $P \leq 0.05$ ) then a transformation was utilized to bring it into conformity with this basic assumption of parametric testing, or the data were subjected to

the Kruskal-Wallis test ( $\chi^2$  approximation) followed, if justified by the statistical probability outcome ( $P \leq 0.05$ ), by Wilcoxon two-sample testing.

## **Results**

### **Distinct weight loss pathologies were elicited by the malnutrition protocols**

Growth indices for the study of IL-10 production in vitro are shown in Table 1, and the wasting disease produced by the two malnutrition protocols was comparable to that of previous studies wherein inflammatory immune competence was depressed [4-6]. Initial body weights did not differ among the dietary groups, whereas final body weights of both malnourished groups were lower than age-matched controls but did not differ from each other. Food intake and carcass composition of the age-matched controls were comparable to previous results pertaining to C57BL/6J weanlings given free access to the complete purified diet used in this investigation [13,14,16,22,24,25,27]. Both malnourished groups exhibited decreased food intakes relative to age-matched controls, as seen previously [6,13,16,25,28]. Further, both malnourished groups exhibited a lower percentage of carcass fat than age-matched controls, and the degree of carcass fat loss was particularly striking in the restricted-intake group. Therefore, the restricted-intake protocol induced a greater decrement in carcass energy than the low-protein system as discussed elsewhere [14,27,29]. Similar outcomes with respect to growth indices were apparent in relation to the second experiment (Table 2) and reflect previous findings [6,13,24] with respect to both the early (day 3) and the more advanced (day 14) stages of weight loss.

### **IL-10 production by mononuclear cells stimulated with LPS in vitro**

Data pertaining to the in vitro production of IL-10 were calculated on a per  $10^5$  mononuclear cells basis and were subjected to a two-way ANOVA (main effects diet and stimulus, the latter discerned by comparing cultures with and without LPS). The outcome revealed no effect of diet ( $P=0.92$ ), an effect of stimulus ( $P<0.0001$ ) and no significant interaction term ( $P=0.84$ ). The magnitude of the 24-hour response to LPS was then assessed by subtraction of the IL-10 concentrations found in the negative control cultures, and this calculation provided a measurement relating to LPS-stimulated IL-10 production above constitutive levels. These data were subjected to a one-way ANOVA which revealed no diet-related effect and the outcome of this analysis is presented in Figure 1.

### **IL-10 capture in vivo following LPS stimulation**

Within the unstimulated controls, there was no effect of diet ( $P=0.46$ ) or stage of weight loss ( $P=0.92$ ). Consequently, the data were combined to yield a correction factor of 253 pg/mL and this was applied to each LPS-stimulated animal. Figure 2, therefore, pertains to LPS-stimulated IL-10 production, only, and reveals that the response to LPS was sustained in the early stage of weight loss (day 3) in both malnourished groups. Conversely, when weight loss was in its advanced stages (day 14), a lower response to LPS was apparent in both malnourished groups relative to age-matched controls and an LPS response was undetectable in the restricted-intake group. Comparison between the two age-matched control groups (day 3 vs. day 14) revealed an ontogeny-associated rise in IL-10 production in response to LPS stimulation ( $P=0.0009$ , least squares means).

By deduction based on the statistical analysis as a whole, the normal ontogenetic rise in LPS-stimulated IL-10 production was attenuated in both forms of acute malnutrition.

Table 1. In vitro response to LPS: Performance outcomes and critical characteristics of weanling mice after 14-day experimental protocol initiated at 19 days of age<sup>1</sup>

Index	Dietary Group <sup>2</sup>			SEM
	C	LP	R	
Initial body weight (g/mouse)	8.3	8.6	8.7	0.09
Final body weight (g/mouse) <sup>3</sup>	18.6 <sup>X</sup>	6.6 <sup>Y</sup>	6.9 <sup>Y</sup>	----
Food intake (g/mouse · d) <sup>4</sup>	2.2 <sup>X</sup>	1.2 <sup>Y</sup>	0.9 <sup>Z</sup>	0.2
Food intake (g/g body weight · d) <sup>5</sup>	0.19 <sup>X</sup>	0.16 <sup>Y</sup>	0.12 <sup>Z</sup>	----
Carcass dry matter (g/100g wet weight)	33.4 <sup>X</sup>	28.1 <sup>Y</sup>	26.8 <sup>Y</sup>	0.6
Carcass lipid (g/100g wet weight)	10.0 <sup>X</sup>	3.9 <sup>Y</sup>	2.7 <sup>Z</sup>	0.2
Mononuclear cells recovered/mouse (x10 <sup>6</sup> ) <sup>6</sup>	50.2 <sup>X</sup>	0.77 <sup>Y</sup>	0.11 <sup>Z</sup>	0.07

<sup>1</sup>Mean values. Within a row, values not sharing a superscript letter differ ( $P \leq 0.05$ ) according to Tukey's Studentized Range test or according to the Kruskal-Wallis procedure followed by Wilcoxon two-sample comparisons.

<sup>2</sup>C: group that consumed complete diet ad libitum; LP: group that consumed low-protein diet ad libitum; R: group that consumed complete diet in restricted daily quantities.

<sup>3</sup>Kruskal-Wallis test of Wilcoxon rank sums that were as follows: C, 93; LP, 29; R, 49.

<sup>4</sup>From ANOVA of inverse-transformed data. Mean values are the inverse of the transformed means.

<sup>5</sup>Kruskal-Wallis test of Wilcoxon rank sums that were as follows: C, 88.0; LP, 62.0; R, 21.0.

<sup>6</sup>Viable mononuclear cells recovered from spleen, mesenteric and inguinal lymph nodes. From ANOVA of natural log-transformed data. Mean values are antilogs of log means.

Table 2. In vivo response to LPS: Performance outcomes and critical composition characteristics of weanling mice after 3-day or 14-day experimental protocols initiated at 19 days of age<sup>1</sup>

Index		Dietary Group <sup>2</sup>			SEM
		C	LP	R	
	<b>Day 3</b>				
Initial body weight (g/mouse)		8.5	8.4	8.6	0.1
Final body weight (g/mouse)		10.4 <sup>X</sup>	7.2 <sup>Y</sup>	7.4 <sup>Y</sup>	0.1
Food intake (g/mouse · d) <sup>3</sup>		2.6 <sup>X</sup>	1.5 <sup>Y</sup>	1.2 <sup>Y</sup>	0.2
Food intake (g/g body weight · d) <sup>3</sup>		0.19 <sup>X</sup>	0.13 <sup>Y</sup>	0.09 <sup>Z</sup>	0.001
Carcass dry matter (g/100g wet weight)		30.3 <sup>X</sup>	28.3 <sup>Y</sup>	27.9 <sup>Y</sup>	0.30
Carcass lipid (g/100g wet weight) <sup>4</sup>		9.2 <sup>X</sup>	5.9 <sup>Y</sup>	3.4 <sup>Z</sup>	0.03
	<b>Day 14</b>				
Initial body weight (g/mouse)		8.3	8.5	8.6	0.08
Final body weight (g/mouse) <sup>4</sup>		17.5 <sup>X</sup>	6.1 <sup>Y</sup>	6.0 <sup>Y</sup>	0.02
Food intake (g/mouse · d) <sup>5</sup>		2.9 <sup>X</sup>	1.3 <sup>Y</sup>	0.9 <sup>Z</sup>	0.01
Food intake (g/g body weight · d)		0.2 <sup>X</sup>	0.1 <sup>Y</sup>	0.08 <sup>Z</sup>	0.002
Carcass dry matter (g/100g wet weight)		32.1 <sup>X</sup>	28.1 <sup>Y</sup>	26.5 <sup>Z</sup>	0.26
Carcass lipid (g/100g wet weight)		10.4 <sup>X</sup>	4.5 <sup>Y</sup>	2.7 <sup>Z</sup>	0.34

<sup>1</sup>Mean values. Within a row, values not sharing a superscript letter differ ( $P \leq 0.05$ )

according to Tukey's Studentized Range test.

<sup>2</sup>C: group that consumed complete diet ad libitum; LP: group that consumed low-protein diet ad libitum; R: group that consumed complete diet in restricted daily quantities.

<sup>3</sup>From ANOVA of squared-transformed data. Mean values are square roots of squared means.

<sup>4</sup>From ANOVA of natural log-transformed data. Mean values are antilogs of log means.

<sup>5</sup>From ANOVA of inverse-transformed data. Mean values are the inverse of the transformed means.

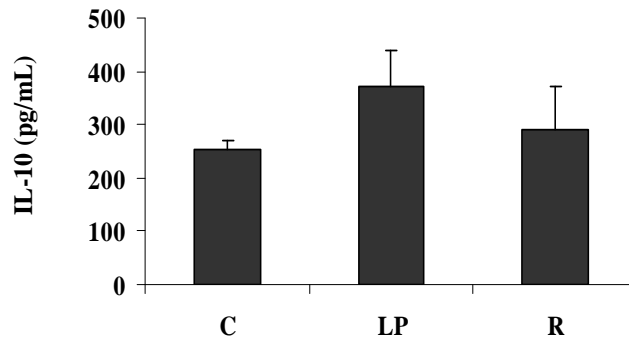


Figure 1. Concentration of IL-10 produced by mononuclear cells from spleen and lymph nodes in response to 24 hours of LPS stimulation in vitro. Each culture included  $10^5$  viable cells and stimulation was achieved using LPS from *Escherichia coli* ( $10 \mu\text{g/mL}$ ). Equal numbers of male and female C57BL/6J mice, initially 19 days old, were fed a complete purified diet ad libitum (group C, age-matched controls,  $n=6$ ), or were given the complete diet in restricted daily quantities (group R,  $n=6$  pooled samples each comprised of 4 mice), or were given free access to an isocaloric low-protein diet (group LP,  $n=6$  pooled samples each comprised of 2 mice). Bars represent means following subtraction of IL-10 concentrations in unstimulated cultures, and the individual SEM values are shown. Data were subjected to a one-way ANOVA ( $P=0.47$ , pooled SEM=36.1).

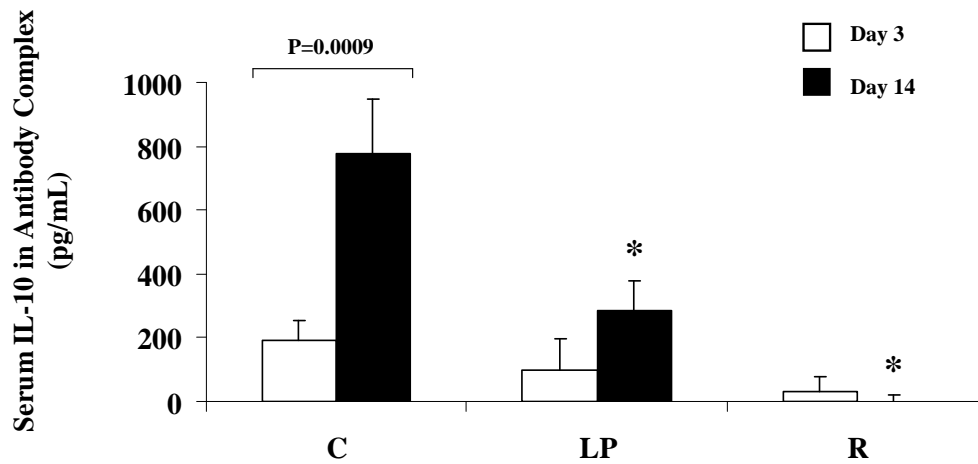


Figure 2. Serum concentration of IL-10 induced by LPS challenge in vivo and complexed with biotin-conjugated anti-IL-10 antibody. Male and female C57BL/6J mice, initially 19-days-old, were fed a complete purified diet ad libitum (group C, age-matched controls), or were given the complete diet in restricted daily quantities (group R), or were given free access to an isocaloric low-protein diet (group LP). Within each feeding period (3 or 14 days), each dietary group included 4 males and 4 females. Blood samples were taken four hours after intraperitoneal injection of 10  $\mu\text{g}$  of a biotin-tagged anti-IL-10 capture antibody together with 1  $\mu\text{g}/\text{gram}$  body weight of *Escherichia coli* LPS. Two-way ANOVA revealed main effects of diet ( $P=0.0001$ ) and stage of weight loss ( $P=0.012$ ) together with a significant interaction term (diet X stage,  $P=0.025$ ), and pooled  $\text{SEM} = 0.80$ . Bars represent mean values (squared means of square root transformed

data), following correction based on unstimulated controls given the capture antibody without LPS challenge, an SEM value is shown for each mean. Within time points, bars marked with an asterisk (\*) differ from age-matched controls ( $P \leq 0.05$ ), according to Least Squares Means.

## **Discussion**

In this investigation, production of IL-10 in response to an inflammatory stimulus was sustained by LPS-responsive cells, on a per cell basis, even in the advanced stages of acute weanling protein and energy deficit. By contrast, IL-10 production by the LPS-responsive compartment, as a whole, was depressed. Inasmuch as these findings pertain to a response to an inflammatory challenge, they represent a second tier of anti-inflammatory regulation complementary to and generated beyond the levels of constitutive production of IL-10 and other hormonal mediators. Moreover, the timeline examined in this work precludes involvement of adaptive immunity, e.g., the thymic-independent humoral response to LPS [30], and centers attention fully on an innate immune response which is mediated through TLR-4 signaling [20]. These findings were apparent in two metabolically distinct murine models of acute pre-pubescent malnutrition, a restricted intake model and a low-protein model, which mimic the well-defined pediatric pathologies of marasmus and incipient kwashiorkor, respectively [6,14,21]. In view of previous findings made using the same experimental systems, second-tier IL-10 production, at least of the innate type, emerges as less robust than constitutive production of this anti-inflammatory cytokine in the face of acute weanling malnutrition [16].

The *in vitro* system used herein yields results that may relate more directly to cytokine production, at least on a per cell basis, than the capture assay. However, because of the non-physiological context of a cell culture microenvironment, the *in vitro* results serve best in a supplementary role. To the extent discernible from this investigation, acute weanling malnutrition imposes no fundamental influence on the

capacity of mononuclear cells to produce IL-10 in an innate-type response to high-dose LPS. Therefore, a simple interpretation of this investigation is that the decline in activation-induced IL-10 production in vivo by the malnourished animals reflects the impact of acute protein and energy deficits on cellular numbers within the LPS-responsive compartment. Lymphoid involution out of proportion with weight loss is a consistent feature of acute pre-pubescent malnutrition [2,31] and encompasses the cellular elements known to both express TLR-4 and produce IL-10, i.e., dendritic cells, mononuclear phagocytes, B cells and activated T cells [32-36]. It remains possible that production of IL-10, on a per cell basis, is affected in vivo. Moreover, a report of modest decreases in expression of LPS receptor complex proteins in acutely protein-deficient mice [37] points to a need for dose-response studies which are beyond the scope of the present investigation. However, a loss of LPS-responsive elements appears sufficient to explain the findings of the present investigation.

The in vivo cytokine capture assay and its interpretation are at the center of this investigation. No in vivo index of cytokine production can be independent of physiological turnover, a potentially crippling confounder in relation to blood cytokines because of their brief half-lives that are generally measured in minutes [38-41]. The capture assay functions by trapping newly-synthesized cytokine in an antibody complex that extends the half-life of the bound cytokine to hours or days in the blood of the mouse [39,41-44]. This assay strategy is simply an extension of the physiological phenomenon whereby the clearance of cytokines from the blood is retarded when these molecules form complexes with soluble cytokine receptors [45] or anti-cytokine autoantibodies [46]. Therefore, when performed with a suitably short assessment period, e.g., four hours as in

the present investigation, the capture assay is not overwhelmed by turnover and yields an index relating to net systemic cytokine production rate in vivo. LPS has previously been utilized successfully in combination with the capture assay [47], and four hours has been demonstrated to provide ample time to generate elevated serum IL-10 concentrations in response to LPS stimulation in vivo [48,49].

The metabolic dissimilarities between mice subjected to the low-protein and restricted-intake protocols, and their respective similarity to the human pathologies of kwashiorkor and marasmus, have been noted elsewhere [21,27,50]. Briefly, the low-protein diet produces weight loss consequent to dietary imbalance, and weanling mice consuming this diet develop fatty liver and edema, the hallmark signs of kwashiorkor, after four weeks or more of wasting disease. When imposed for the periods of time reported in this investigation, therefore, the low-protein protocol may be considered to produce an incipient kwashiorkor. It is noteworthy, also, that the weight loss produced by this protocol emphasizes loss of lean tissue with relative preservation of fat (at least relative to the restricted-intake model), as would be anticipated in a model relevant to kwashiorkor. In contrast, the restricted-intake protocol induces weight loss apart from dietary imbalance. This protocol stimulates the extreme mobilization of fat reserves that would be expected in a model of marasmus but fails to produce the hepatic pathology seen in mice subjected to the low-protein protocol. Despite their metabolic differences, these forms of experimental malnutrition have consistently failed to exhibit distinct immunological characteristics [3,14,21,23,25,27,28] and the findings of the present investigation sustain this pattern.

Recently, the profoundly depressed inflammatory competence consistently associated with acute pre-pubescent malnutrition has been proposed to result from a tightly regulated shift in the physiological balance between inflammatory and anti-inflammatory immune competence [15,16]. In this model, maintaining an anti-inflammatory form of immune competence reduces the risk of autoimmune disease in the face of catabolic challenge. This proposition is the antithesis of the classical and prevalent model in which inflammatory defences are lost in a chaotic and disintegrative manner as weight loss progresses, and the model is founded on studies of the constitutive production and concentrations of both inflammatory and anti-inflammatory cytokines and hormones in acute weanling malnutrition [13-16,25,28]. Constitutive production of anti-inflammatory mediators including IL-10 determines the microenvironment that prevails when an inflammatory stimulus is first encountered. This primary tier of anti-inflammatory defence is sustained even into the advanced stages of weight loss in diverse forms of acute weanling protein and energy deficit [16]. By contrast, the present investigation reveals that the second tier of anti-inflammatory defence represented by IL-10 production, at least of an innate type, may be less robust and declines in the latter stages of the progression of weight loss. Ultimately, all aspects of immune competence must succumb to disintegrative loss if wasting disease is permitted to progress indefinitely. Hence, it is noteworthy that the classic disintegrative model accommodates the findings reported herein pertaining to malnutrition-associated loss of second-tier anti-inflammatory capacities, a decrement that appears primarily attributable to a failure of normal ontogeny. Loss of this component of anti-inflammatory competence, or its failure to develop according to the normal physiological pattern, may not immediately

compromise the physiological balance of immune defences but may provide an early sign that the ability to sustain a regulated anti-inflammatory form of immune competence is beginning to fail.

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## **Chapter 8**

**The capacity for interleukin-10 production by the effector/memory T cell compartment of the weanling mouse responds differently to distinct forms of acute malnutrition**

## **Abstract**

Constitutive interleukin(IL)-10 production is sustained into the advanced stages of acute (i.e., wasting) pre-pubescent protein-energy malnutrition. A second tier of IL-10-centered immune regulation includes both innate and adaptive responses to inflammatory stimuli, and the influence of acute weanling malnutrition on the adaptive-type second-tier response was assessed herein. Male and female C57BL/6J mice, initially 19 days old, consumed a complete diet ad libitum, an isocaloric low-protein diet ad libitum (mimicking incipient kwashiorkor), or the complete diet in restricted daily quantities (mimicking marasmus). After 14 days, IL-10 concentrations generated by exposure of splenic and nodal T cells to plate-bound anti-CD3 for 24 hours were unaffected by malnutrition ( $P=0.32$ ) or by a second stimulus from anti-CD28 ( $P>0.05$ ). Additionally, systemic IL-10 production over 4 hours following injection of anti-CD3 (10  $\mu\text{g}$  per mouse) was assessed using the in vivo cytokine capture assay at early (3 days) and advanced (14 days) stages of weight loss. IL-10 production in response to anti-CD3 in vivo was sustained into advanced weight loss by the T cell compartment of the low-protein group ( $P>0.05$ ), but exhibited a depression in the restricted-intake group ( $P<0.05$ ). Decline in second-tier IL-10 production by the effector/memory T cell compartment may constitute an early sign that non-inflammatory control over immune competence is beginning to disintegrate in wasting malnutrition.

**Keywords:** anti-inflammatory, cytokine, interleukin-10 protein-energy malnutrition, mouse

## **Introduction**

Up to two million largely infection-related deaths occur annually among children under five years of age suffering from acute deficits of protein and energy, and this toll is undoubtedly exceeded by the additional burden of infection-related morbidity [1]. In its most severe forms, acute pre-pubescent malnutrition manifests in two distinct pathologies, namely marasmus and kwashiorkor, and a defining characteristic common to both is a depression in inflammatory immune competence with consequent susceptibility to opportunistic infections [2,3]. Malnutrition-associated immune depression is widely attributed to a disintegrative loss of inflammatory capacities, but this paradigm is under challenge by a proposition centered on active, anti-inflammatory immune regulation [2,4,5]. Briefly, the so-called “tolerance model” identifies malnutrition-related immune depression as a physiological strategy to reduce the risk of autoimmune disease in the face of catabolically-released self antigens. The model acknowledges the resulting cost in terms of susceptibility to infection [6,7] and accommodates the biologically trivial endpoint that a disintegrative loss of all forms of immune competence must ultimately prevail if wasting disease is permitted to proceed indefinitely.

The tolerance model centers on three anti-inflammatory mediators, namely the glucocorticoids, transforming growth factor(TGF)- $\beta$  and interleukin(IL)-10. This hormonal triad plays a determining role in physiological anti-inflammatory control and the maintenance of self tolerance [8-13], and the constitutive blood levels of these mediators collectively represent the primary or first tier of anti-inflammatory and regulatory immune competence which effectively serves in a sentinel role, positioned to respond when an inflammatory stimulus is first encountered. Recently, high constitutive

blood levels of each member of this anti-inflammatory triad, have been reported in weanling mouse models of marasmus and incipient kwashiorkor which consistently reproduce the critical features of these pediatric pathologies [4-6,14]. Further, remarkably, the systemic rate of constitutive IL-10 production is at least sustained even into the advanced stages of wasting pathology in the same high-fidelity mouse models of pre-pubescent malnutrition [6].

In addition to their constitutive production, anti-inflammatory mediators are released as a component of any competent response to inflammatory stimuli [15], and the concept of layers of anti-inflammatory immune regulation has been previously identified [16]. Thus, stimulus-dependent production may be regarded as a second tier of anti-inflammatory and tolerogenic immune regulation, and both innate and adaptive types of second-tier responses can be identified. IL-10 is a particularly important anti-inflammatory mediator that regulates both innate and adaptive immune responses [17,18]. Recent evidence indicates that the innate type of second-tier IL-10 production is less robust than constitutive production of this cytokine in diverse forms of acute weanling protein and energy deficit [7]. However, information is unavailable pertaining to second-tier IL-10 production by adaptive immune cellular components. T cells are an important source of IL-10 in the context of an adaptive response [18]. Therefore, the objective of this investigation was to assess, both *in vitro* and *in vivo*, the influence of metabolically distinct forms of acute malnutrition on adaptive-type second-tier IL-10 production by the T cell compartment of the weanling mouse in models known to sustain constitutive production of this anti-inflammatory cytokine.

## **Materials and Methods**

### *Animals and housing facilities*

Male and female C57BL/6J mice were obtained from an in-house breeding colony derived from animals originally purchased from the Jackson Laboratory (Bar Harbor, ME). Caging and housing conditions were exactly as described previously [19-21], and this investigation was approved by the Animal Care Committee of the University of Guelph in accordance with the Canadian Council on Animal Care.

### *Diets and experimental design*

Eighteen-day-old animals were weaned and acclimated overnight to a complete purified diet described in detail elsewhere [22]. Afterward, equal numbers of male and female 19-day-old mice were randomly assigned to one of three experimental groups, namely an age-matched control group consuming the complete purified diet ad libitum, a restricted-intake group consuming the complete purified diet in restricted daily quantities based on calculations that relate ad libitum food intake (g food/g body weight) to chronological age in the weanling mouse [21], or a low-protein group consuming ad libitum a nitrogen-deficient purified diet that was isocaloric with the complete formulation [4,23]. The restricted-intake protocol produces a pathology that reproduces the critical features of pediatric marasmus, whereas the low-protein model closely resembles incipient pediatric kwashiorkor [4,14,21,23-25].

The capacity of the T cell compartment to respond to T cell receptor-mediated stimulation was assessed both in vitro and in vivo. Two experiments were performed in which the three dietary groups were maintained on their respective regimens for 14 days,

i.e. from 19 through 33 days of age. Additionally, in the second experiment a cohort of animals was examined after only 3 days (i.e., at 22 days of age), a time point taken to represent early-stage weight loss [5,6,14].

In the first experiment, a sample size of ten was achieved for the age-matched control group and a sample size of eight was achieved for the restricted-intake and low-protein groups. In order to obtain sufficient numbers of cells from secondary lymphoid organs (spleen combined with mesenteric and inguinal lymph nodes), pooled samples were required for mice subjected to the low-protein and restricted-intake groups. Each pooled sample (2-4 mice for the low-protein group and 2-8 mice for the restricted-intake group) included equal numbers of males and females and constituted a single degree of freedom for the purpose of statistical analysis. The experiment was conducted according to a 2 X 2 design in which diet and in vitro stimulation of T cells using anti-CD3 were the main effects and cells were harvested for study at the end of the 14-day experimental period. In the second experiment, eight mice (four males and four females) were included within each experimental group at each time point assessed, and pooling was unnecessary. In vivo capture of IL-10 following a single injection of anti-CD3 was assessed using blood samples taken at day 3 and day 14 (early and advanced weight loss, respectively), thus yielding a 2 X 2 design in which the main effects were diet and stage of weight loss. Carcasses from animals of both experiments were stored at -20°C to await analysis.

*Procedure to obtain mononuclear cell suspensions for in vitro stimulation*

After measurement of body weight, mice were anesthetized with CO<sub>2</sub> and killed by cervical dislocation without recovering consciousness. The spleen and the mesenteric and inguinal lymph nodes were removed aseptically, diced together and forced through a sterile stainless steel wire screen (100-mesh) into RPMI 1640 medium (Flow Laboratories, Mississauga, Canada) containing 10% heat-inactivated fetal bovine serum (Sigma Chemical, St. Louis, MO), 1 mmol/L HEPES (ICN Biomedicals, Aurora, OH), 10<sup>5</sup> U/L penicillin and 100 mg/L streptomycin (hereafter designated “complete medium”). A single-cell suspension of mononuclear cells was produced by discontinuous gradient centrifugation as described previously [19]. Cell numbers were determined using a hemocytometer and viability, assessed by eosin Y exclusion, always exceeded 95%.

*In vitro stimulation of T cells to elicit IL-10 production*

Falcon plates (Becton Dickinson Labware, New Jersey, USA, catalog #3072) were coated overnight at 4°C with 200 µL 0.01 M phosphate-buffered saline (PBS, pH 7.3) containing 5 µg/mL anti-CD3 (clone: 145-2C11; Cedarlane Laboratories, Hornby, Canada). After coating, plates were washed twice with PBS and each well received 2 x 10<sup>5</sup> viable mononuclear cells in 190 µL complete medium. Subsequently, 10 µL of PBS was added to half of the culture wells while the remaining test cultures received 10 µL of PBS containing anti-CD28 (clone: 37.51.1; Cedarlane Laboratories, Hornby, Canada) to achieve a final concentration of 20 µg/mL. Negative control cultures were produced using wells not coated with anti-CD3 and the cells were cultured in fluids comprising 190

$\mu$ L complete medium together with 10  $\mu$ L PBS. All cultures were incubated at 37°C for 24 h. After incubation, plates were centrifuged for 1 min at 200 x g, and supernatants from wells containing cultures of the same dietary group and stimulus were pooled and stored at -80°C.

*Assay of IL-10 concentrations generated in vitro*

A sandwich ELISA kit for assay of mouse IL-10 (BD Biosciences, Mississauga, ON, catalogue #555252) was applied to samples of culture fluids as described by the manufacturer. Outcomes were quantified by optical density using a Vmax kinetic plate reader (Molecular Devices Corp., Menlo Park, CA) set for absorbance at 450 nm with wavelength correction based on absorbance at 570 nm. Only the linear portions of standard curves were used (mean  $R^2 = 0.99$ ), and all samples fell within this part of the curve. The reliability (intra-assay coefficient of variation) averaged 8.1% and its detection limit, determine as described elsewhere [26], averaged 1.2 pg/mL.

*Assessment of percentage CD3<sup>+</sup> cells in mononuclear cell suspensions from spleen and lymph nodes*

Analyses were performed using a Becton-Dickinson FACSCalibur flow cytometer equipped with BD CellQuest TM software (2001). Generic aspects of staining procedures in this laboratory, including Fc receptor blockade, are described elsewhere [19,21]. Cells were subjected to single-color analysis by means of a phycoerythrin-conjugated anti-mouse CD3 $\epsilon$  monoclonal antibody (clone: 145-2C11, Armenian hamster IgG, eBiosciences, San Diego, CA) at a concentration of 0.2  $\mu$ g per 250 x 10<sup>3</sup> viable

cells. Negative control samples were stained with biotin-conjugated hamster IgG (Cedarlane Laboratories, Hornby, ON) followed by phycoerythrin-conjugated streptavidin (Cedarlane Laboratories, Hornby, ON), respectively at concentrations of 0.2  $\mu\text{g}$  and 0.15  $\mu\text{g}$  per  $250 \times 10^3$  viable cells. Cells were incubated with staining reagents in the dark on ice for 40 min, and were fixed with paraformaldehyde (20 g/L) after which samples were analyzed within 7 days. Viability before staining was determined by eosin Y exclusion and always exceeded 95%. Each analysis was based on at least  $10^4$  events after dead cells and residual erythrocytes were eliminated by gating on the basis of forward-angle light scatter.

#### *In vivo IL-10 capture assay*

An in vivo cytokine capture assay kit for murine IL-10 (BD Biosciences, Mississauga, Canada, catalogue # 558072) was utilized as described previously [6]. Briefly, each animal received an aseptic intraperitoneal injection of 10  $\mu\text{g}$  of biotin-conjugated anti-mouse IL-10 (“capture”) antibody delivered in 200  $\mu\text{L}$  of endotoxin-free physiological saline (Vétoquinol N.-A. Inc., Lavaltrie, Québec; henceforth, “saline”) together with 10  $\mu\text{g}$  of anti-mouse CD3 (clone: 145-2C11, Armenian hamster IgG; BD Bioscience, San Diego, CA). Isotype controls received 10  $\mu\text{g}$  of capture antibody plus 10  $\mu\text{g}$  of Armenian hamster IgG (catalog # G235-2356, BD Biosciences, San Diego, CA, USA) by intraperitoneal injection in 200  $\mu\text{L}$  of sterile saline (n=3, 2 males and 1 female in each dietary group and stage of weight loss). Blood samples were collected 4 hours later to permit analysis of the concentration of IL-10 that had accumulated in molecular complexes with the capture antibody. A sandwich ELISA was used to detect the

antibody/cytokine complexes and was performed with reagents provided in the capture assay kit (BD Biosciences). The capture reagent of the ELISA was a rat monoclonal antibody that recognized a different IL-10 epitope than the biotin-conjugated capture antibody used *in vivo*, and the detection reagent was streptavidin conjugated with horseradish peroxidase. In a minor departure from the manufacturer's instructions, an additional blocking step was included using 10% fetal bovine serum (Sigma Chemical) in sterile PBS (1.0 mM, pH 7.3) following the capture stage of the ELISA. Outcomes were quantified by optical density using a Vmax kinetic plate reader (Molecular Devices Corp., Menlo Park, CA) set for absorbance at 450 nm with wavelength correction based on absorbance at 570 nm.

The reliability (intra-assay coefficient of variation) and detection limit of each assay were estimated as described previously [26] and averaged 4.3% and 0.14 pg/mL (of IL-10 in molecular complex) respectively. The standard curves for the assay were linear (mean  $R^2 = 0.99$ ) over a concentration range up to 2000 pg/mL. All samples fell within the linear portion of the curve and exceeded the detection limit of the assay.

#### *Blood sampling procedure for the in vivo capture assay*

As described previously [14] blood samples were collected from the orbital plexus while the mice were under CO<sub>2</sub> anesthesia, and the mice were then killed by cervical dislocation without recovering consciousness. Blood was allowed to clot at room temperature for 30 - 45 minutes and the resulting serum was stored at -80°C to await analysis.

### *Carcass Composition*

Carcasses from animals of both experiments were stored at -20<sup>0</sup>C to await analysis. Analysis of dry matter and total lipid content was performed as described previously [14,21].

### *Statistical Analysis*

Statistical analyses were conducted using the SAS system (SAS Institute, Cary, NC) for Windows (version 9.0), and a predetermined upper limit of probability of  $P \leq 0.05$  was applied for statistical significance. Data were subjected to a two-way ANOVA followed, if justified by the level of statistical probability ( $P \leq 0.05$ ), by Tukey's Studentized Range test. If a data set failed to exhibit normal distribution according to each of the four tests applied by the SAS program ( $P \leq 0.05$ ) then a transformation was utilized to bring it into conformity with this basic assumption of parametric testing.

## **Results**

### *Distinct Weight Loss Pathologies Were Elicited by the Malnutrition Protocols*

Growth indices for the primary interest of this investigation, anti-CD3 stimulated IL-10 production in vivo, are shown in Table 1. Initial body weights did not differ among the dietary groups and body weight gain, food intake and carcass composition of the age-matched control group were comparable to previous results pertaining to C57BL/6J weanlings given free access to the complete diet used in this investigation at both time points [4,6,14,22-24,27]. The final body weights of the malnourished groups were lower than those of their corresponding age-matched control groups but did not

differ from each other. Moreover, the weight loss produced by the two malnutrition protocols was comparable to that reported in previous studies wherein inflammatory immune competence was depressed [20,21,28]. In addition, the malnourished groups exhibited decreased food intakes relative to their corresponding age-matched controls, as seen previously [6,14,21,23,29]. Further, both malnourished groups exhibited a lower percentage of carcass fat than controls, but the degree of carcass fat loss was more pronounced in the restricted-intake group at both time points assessed. Therefore, as reported elsewhere [4,24,30], the restricted-intake protocol induced a greater decrement in carcass energy than the low-protein protocol, and this was apparent even at an early stage of weight loss. Similar outcomes with respect to growth indices were apparent in the experiment centered on the *in vitro* response to anti-CD3 stimulation (results not shown).

#### *IL-10 production by mononuclear cells stimulated *in vitro* with anti-CD3*

The percentage of (CD3<sup>+</sup>) T cells recovered from the spleen and lymph nodes was higher in the low-protein group than in the age-matched controls (means: 44.7% and 29.9%, respectively), whereas the restricted-intake group (mean: 39.0%) did not differ from either of the other two dietary groups (Tukey's Studentized Range test, pooled SEM = 3.36). These findings were comparable to outcomes reported previously in the same experimental systems [24,25]. The percentage of T cells in mononuclear cell populations of secondary lymphoid organs is consistently high relative to age-matched controls in mice fed the low-protein diet [24,25]. By contrast, the restricted-intake protocol affects

this index only inconsistently [24,25], and failed to affect it in the present investigation. A representative flow cytometry histogram is shown in Figure 1.

The concentration of IL-10 in culture fluids was expressed per  $10^5$  CD3<sup>+</sup> mononuclear cells, and analysis of these data by two-way ANOVA revealed an effect of stimulus (P=0.0081) but no significant effect of diet (P=0.16) or interaction (P=0.70). With respect to the effect of stimulus, plate-bound anti-CD3 increased IL-10 production (when compared to PBS) but addition of anti-CD28 to provide a second stimulus made no difference to IL-10 concentrations relative to stimulation with anti-CD3, alone (Tukey's Studentized Range test, P>0.05). The magnitude of the 24-hour response to anti-CD3 stimulation was then determined by combining the outcomes of stimulated cultures independently of the involvement of the CD28 receptor and by subtraction of the IL-10 concentrations found in corresponding negative control (PBS) cultures. This calculation provided a measurement relating to anti-CD3-stimulated IL-10 production by T cells above constitutive levels, and the data were subjected to a one-way ANOVA. As shown in Figure 2, no diet-related difference emerged in the response expressed on a per  $10^5$  CD3<sup>+</sup> cell basis.

#### *IL-10 capture in vivo following stimulation with anti-CD3*

No effect of diet (P=0.28) or stage of weight loss (P=0.38) was apparent among the isotype control groups, and their average serum level of antibody-captured IL-10 was 297 pg/mL. Therefore this correction factor was applied to each anti-CD3-stimulated animal to ascertain the magnitude of the systemic IL-10 response to polyclonal T cell stimulation. Two-way ANOVA revealed a diet-related effect (P=0.016) but no effect of

the stage of weight loss ( $P=0.70$ ) and no interaction term ( $P=0.26$ ). This outcome pertains to anti-CD3-stimulated IL-10 production, only, and is represented in Figure 3. The 4-hour accumulation of IL-10 in the blood of the restricted-intake group was depressed, independently of the stage of weight loss, relative to age-matched controls. Conversely, the response of the low-protein group to polyclonal T cell stimulation did not differ from that of the age-matched controls.

Table 1. Study of response to anti-CD3 stimulation in vivo: performance outcomes after 3-day and 14-day experimental protocols initiated at 19 days of age<sup>1</sup>

Index	Dietary Group <sup>2</sup>			SEM
	C	LP	R	
<b>Day 3</b>				
Initial body weight (g/mouse)	8.4	8.5	8.7	0.09
Final body weight (g/mouse)	10.1 <sup>X</sup>	7.4 <sup>Y</sup>	7.5 <sup>Y</sup>	0.12
Food intake (g/mouse · 3 d) <sup>3</sup>	7.5 <sup>X</sup>	4.9 <sup>Y</sup>	3.5 <sup>Z</sup>	0.04
Food intake (g/g body weight · d) <sup>4</sup>	0.18 <sup>X</sup>	0.13 <sup>Y</sup>	0.09 <sup>Z</sup>	0.24
Carcass dry matter (g/100g wet weight) <sup>3</sup>	30.4 <sup>X</sup>	29.6 <sup>X</sup>	27.4 <sup>Y</sup>	0.01
Carcass lipid (g/100g wet weight)	8.0 <sup>X</sup>	6.1 <sup>Y</sup>	3.8 <sup>Z</sup>	0.23
<b>Day 14</b>				
Initial body weight (g/mouse)	8.8	8.6	8.8	0.10
Final body weight (g/mouse) <sup>3</sup>	18.5 <sup>X</sup>	6.5 <sup>Y</sup>	6.2 <sup>Y</sup>	0.02
Food intake (g/mouse · 14 d) <sup>3</sup>	65.6 <sup>X</sup>	16.6 <sup>Y</sup>	11.2 <sup>Z</sup>	0.03
Food intake (g/g body weight · d) <sup>3</sup>	0.27 <sup>X</sup>	0.10 <sup>Y</sup>	0.07 <sup>Z</sup>	0.02
Carcass dry matter (g/100g wet weight) <sup>3</sup>	32.8 <sup>X</sup>	27.9 <sup>Y</sup>	26.8 <sup>Y</sup>	0.02
Carcass lipid (g/100g wet weight) <sup>3</sup>	10.0 <sup>X</sup>	4.1 <sup>Y</sup>	2.0 <sup>Z</sup>	0.04

<sup>1</sup>Mean values. Within a row, values not sharing a superscript letter differ ( $P \leq 0.05$ ) according to Tukey's Studentized Range test.

<sup>2</sup>C: group that consumed complete diet ad libitum; LP: group that consumed low-protein diet ad libitum; R: group that consumed complete diet in restricted daily quantities.

<sup>3</sup>From ANOVA of natural log-transformed data. Mean values are antilogs of log means.

<sup>4</sup>From ANOVA of inverse-transformed data. Mean values are the inverse of the transformed means.

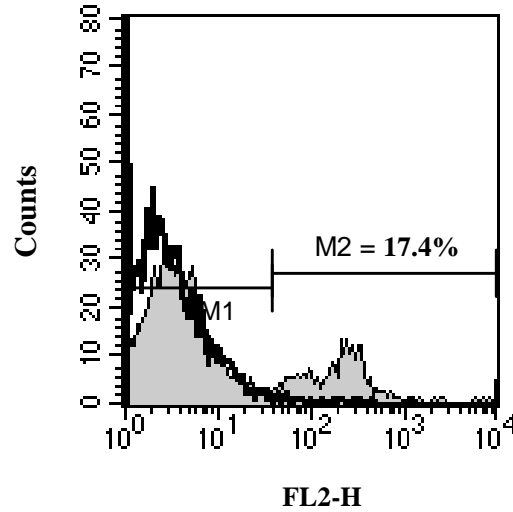


Figure 1. Overlay of flow cytometric histograms to identify CD3 $\epsilon$ <sup>+</sup> mononuclear cells from combined spleen, inguinal and mesenteric lymph nodes of a 33-day-old C57BL/6J mouse given free access to the complete purified diet for 14 days beginning at 19 days of age. 250 x 10<sup>3</sup> cells were stained with phycoerythrin-conjugated anti-mouse CD3 $\epsilon$  (145-2C11, hamster IgG, solid grey histogram) or with biotin-conjugated hamster IgG followed by phycoerythrin-conjugated streptavidin (clear histogram). The number of fluorescent events is shown in arbitrary units on the Y axis and fluorescence intensity is represented in arbitrary base 10 logarithmic units on the X axis. The stained population is subdivided to distinguish CD3 $\epsilon$ <sup>+</sup> cells (M2) from cells not expressing this marker (M1), and the percentage of positive cells is shown. Each histogram in the overlay represents an analysis of 10<sup>4</sup> fluorescent events,

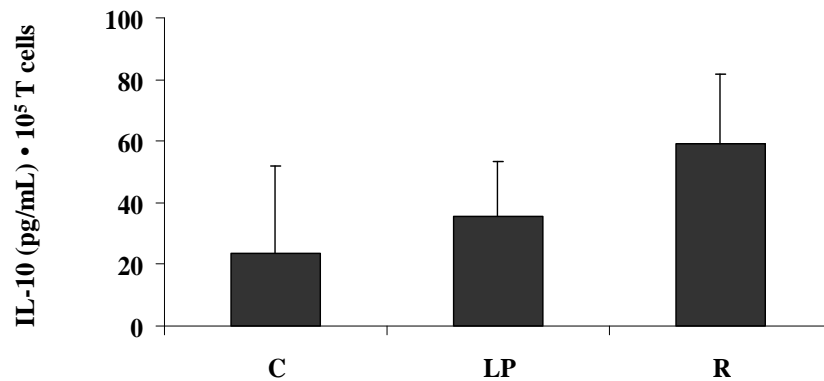


Figure 2. Concentration of IL-10 found in cultures of mononuclear cells from the spleen and lymph nodes after 24 hours of stimulation in vitro. Each culture well included  $2 \times 10^5$  viable cells. Polyclonal stimulation was achieved with plate-bound anti-CD3 ( $5 \mu\text{g/mL}$ ), and soluble anti-CD28 ( $20 \mu\text{g/mL}$ ) was included in half of the cultures subjected to mitogen. Unstimulated cultures were exposed to PBS in place of mitogen. Equal numbers of male and female C57BL/6J mice, initially 19 days old, were fed a complete purified diet ad libitum (group C, age-matched controls,  $n=10$ ), or were given the complete diet in restricted daily quantities (group R,  $n=8$  pooled samples each comprised of 2-8 mice), or were given free access to an isocaloric low-protein diet (group LP,  $n=8$  pooled samples each comprised of 2-4 mice). Each pooled sample was assigned a single degree of freedom for the purpose of statistical analysis. Within each dietary group, data were normalized on a per  $10^5$  T cell basis and data from stimulated cultures (anti-CD3  $\pm$

anti-CD28) were corrected by subtraction of the IL-10 level found in unstimulated cultures. Data were subjected to a one-way ANOVA ( $P=0.32$ , pooled SEM = 0.18). Bars represent anti-logs of means from natural log-transformed data, and SEM is shown for each mean value.

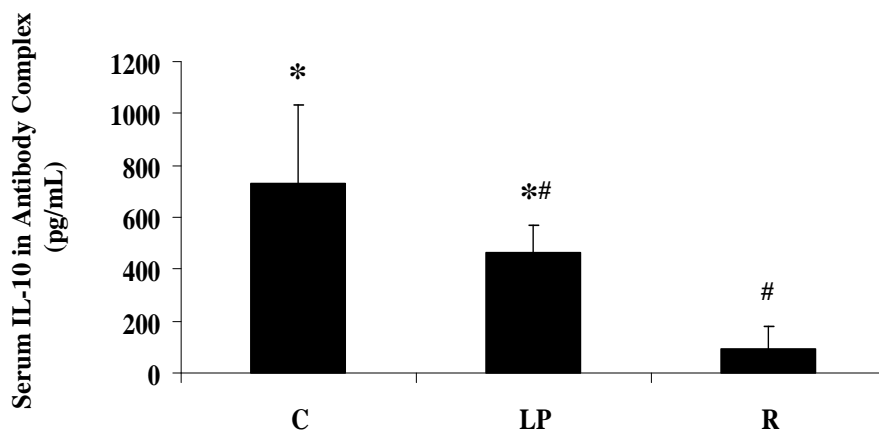


Figure 3. Anti-CD3-induced serum concentration of IL-10 in complex with biotin-conjugated anti-IL-10 antibody, corrected by subtraction of the values found in isotype controls. Blood samples were taken from C57BL/6J mice four hours after intraperitoneal injection of 10  $\mu$ g of the biotin-tagged capture antibody plus 10  $\mu$ g of anti-CD3 (145-2C11). Male and female mice, initially 19 days old, were fed a complete purified diet ad libitum (group C, age-matched controls), or were given the complete diet in restricted daily quantities (group R), or were given free access to an isocaloric low-protein diet (group LP). Within each feeding period (3 or 14 days), n=8/dietary group (4 males and 4 females). Two-way ANOVA revealed a diet main effect (P=0.016) but no effect of time (i.e., stage of weight loss, P=0.70) or interaction (diet x time, P=0.26) and the pooled S.E.M. was 1.6. Bars represent mean values (squared means of square root transformed data), following correction based on isotype controls given 10  $\mu$ g of the capture antibody plus 10  $\mu$ g of Armenian hamster IgG. A SEM value is shown for each mean. Bars not

sharing a symbol differ from each other ( $P \leq 0.05$ ), according Tukey's Studentized Range test.

## Discussion

The present investigation centered on the production of IL-10 by T cells in response to polyclonal stimulation through the T cell receptor in metabolically distinct forms of acute malnutrition in the weanling mouse. Specifically, the low-protein model reproduces the critical features of incipient kwashiorkor as seen in pre-pubescent humans and the restricted-intake model mimics childhood marasmic pathology [19-21,28]. On a per T cell basis, anti-CD3-stimulated IL-10 production in vitro was sustained in the advanced stages of both forms of malnutrition when compared to age-matched controls. By contrast, anti-CD3-induced IL-10 production by the T cell compartment as a whole, in vivo, was sustained in the low-protein group but depressed in the restricted-intake group, independently of the stage of weight loss. These findings pertain specifically to the effector/memory subset of T cells which is a major source of IL-10 within the adaptive system of immune defence. Thus, the response assessed herein represents the magnitude of second-tier anti-inflammatory regulation, i.e. over and above constitutive activity, of an adaptive immune type. In the face of diverse forms of acute pre-pubescent malnutrition, constitutive IL-10 production is robust [6], whereas the capacity for innate-type second-tier IL-10 production is less well sustained [7]. In contrast, the capacity for adaptive-type second-tier IL-10 production appears to respond in a manner determined by the type of malnutrition pathology. This outcome is most readily compatible with the tolerance hypothesis of malnutrition-associated inflammatory decline.

A cytokine response attributable to the effector/memory T cell compartment was isolated for study both in vitro and in vivo in this investigation. To achieve effector status, naïve T cells require more than the short response periods studied in the present

investigation [31,32]. Further, naïve T cells cannot differentiate to effector status if stimulated only through the T cell receptor [33]. However, in the present investigation, the *in vivo* response was elicited exclusively by stimulation through the CD3 molecule and, *in vitro*, co-stimulation through CD28 failed to increase the production of end-stage cytokines. Consequently the findings reported herein reflect the second tier IL-10-centered regulatory potential of the T cell effector compartment apart from the confounding contribution by newly activated naïve T cells.

The *in vitro* system reveals that acute weanling malnutrition imposes no fundamental change in the capacity of effector/memory T cells to produce IL-10, but these findings serve best in a supplementary role to the results of the *in vivo* capture assay which lie at the center of this investigation. No *in vivo* index of cytokine production can be independent of physiological turnover, a potentially crippling confounder in relation to blood cytokines because of their brief half-lives that are generally measured in minutes [34-37]. The capture assay functions by trapping newly-synthesized cytokine in an antibody complex that extends the half-life of the bound cytokine to hours or days in the blood of the mouse [35,37-40]. This assay strategy is simply an extension of the physiological phenomenon whereby the clearance of cytokines from the blood is retarded when these molecules form complexes with soluble cytokine receptors [41] or anti-cytokine autoantibodies [42]. Therefore, when performed with a suitably short assessment period, as in the present investigation, the capture assay is not overwhelmed by turnover and yields an index relating to net systemic cytokine production rate *in vivo*.

The metabolic dissimilarities between mice subjected to the low-protein and restricted-intake protocols, and their respective similarity to the human pathologies of kwashiorkor and marasmus, have been noted elsewhere [19,24,43]. Briefly, the low-protein diet produces weight loss consequent to dietary imbalance, and weanling mice consuming this diet develop fatty liver and edema, the hallmark signs of kwashiorkor, after four weeks or more of wasting disease. When imposed for the periods of time reported in this investigation, therefore, the low-protein protocol produces an incipient kwashiorkor. In contrast, the restricted-intake protocol induces weight loss apart from dietary imbalance. This protocol stimulates the extreme mobilization of fat reserves that would be expected in a model of marasmus but fails to produce the hepatic pathology seen in mice subjected to the low-protein protocol. Further, the restricted-intake system produces a vigorously gluconeogenic animal whereas the low-protein diet, with its high level of digestible carbohydrate, has the opposite metabolic influence [43,44]. Despite their qualitative metabolic differences, these forms of experimental malnutrition have consistently exhibited immunological (including cytokine) characteristics that differ only quantitatively [2,4,19,23-25,30]. The findings reported herein, however, reveal an early-stage immunological decline in a model of pre-pubescent marasmus that was not apparent even in the advanced stages of a model of incipient kwashiorkor. It is interesting that an anti-inflammatory capacity appears preferentially sustained in a form of protein and energy deficit that emphasizes mobilization of lean tissue which is much richer in self antigens, weight-for-weight, than fat reserves.

The tolerance model of malnutrition-associated immune depression proposes a purposeful anti-inflammatory immune regulation that is sustained despite a limited

supply of energy and substrates and that is, as such, the antithesis of the classic paradigm of chaotic immunological disintegration [2,4]. Ultimately, all aspects of immune competence must succumb to disintegrative loss if wasting disease is permitted to progress indefinitely. However, constitutive IL-10 production is sustained into the advanced stages of weanling malnutrition [6], and the present investigation extends this phenomenon of robust anti-inflammatory potential to include adaptive-type second-tier production of IL-10 in a model of incipient kwashiorkor. This finding is in notable contrast to the inability to sustain this form of anti-inflammatory competence in a model of pre-pubescent marasmus. Loss of anti-inflammatory capacities may provide an early sign that the ability to regulate immune competence is beginning to fail. More fundamentally, however, the finding that the capacity to sustain T cell IL-10 production in acute malnutrition is diet-related in a manner independent of nitrogen or energy decrement is more easily accommodated by the tolerance model than by a model centered on a biologically trivial disintegrative loss of inflammatory competence.

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## **Chapter 9**

**Elevated interleukin-10 gene expression in the spleen of acutely protein and energy malnourished weanling mice**

## Abstract

The advanced stages of acute (i.e., wasting) protein energy malnutrition exhibit elevated blood and tissue fluid levels of the anti-inflammatory hormone, interleukin(IL)-10. Further, the systemic IL-10 production rate is at least sustained into the advanced stages of acute malnutrition. To ascertain anatomical sites likely to contribute to the aforementioned phenomenon, our objective was to determine the IL-10 producing potential in several major lymphoid organs. Male and female C57BL/6J mice, initially 19 days of age, consumed a complete purified diet either ad libitum (age-matched controls) or in restricted daily quantities (restricted-intake group, mimicking marasmus), or consumed a purified isocaloric low-protein diet ad libitum (low-protein group, mimicking incipient kwashiorkor) for 14 days. IL-10-producing potential was assessed by real-time quantitative PCR in the spleen, bone marrow and small intestine. The expression of IL-10 mRNA in the spleen, relative to the expression of  $\beta$ -actin, was 6-fold higher in the restricted-intake group ( $P=0.039$ ) and 2-fold higher in the low-protein group ( $P=0.045$ ) than was found in the age-matched control group. Additionally, the malnourished groups sustained IL-10 mRNA expression in both the bone marrow and small intestine in comparison to age-matched controls ( $P>0.05$ ). Thus, the spleen emerges as a secondary lymphoid organ likely to contribute to the high blood and tissue fluid levels of IL-10 reported elsewhere in advanced stages of acute weanling malnutrition. The findings of this investigation provide a mechanistic basis for the proposition that the depressed inflammatory competence characteristic of acute malnutrition represents a regulated pathophysiology.

## **Introduction**

Up to two million largely infection-related deaths occur annually among children under five years of age suffering from acute deficits of protein and energy, and this toll is undoubtedly exceeded by the additional burden of infection-related morbidity [1]. In its most severe forms, acute pre-pubescent malnutrition manifests in two distinct pathologies, namely marasmus and kwashiorkor, and a defining characteristic common to both is a depression in inflammatory immune competence with consequent susceptibility to opportunistic infections [2,3]. Malnutrition-associated immune depression is widely attributed to a disintegrative loss of inflammatory capacities, but this paradigm is under challenge by a proposition centered on active, anti-inflammatory immune regulation [2,4,5]. Briefly, the so-called “tolerance model” identifies malnutrition-related immune depression as a physiological strategy to reduce the risk of autoimmune disease in the face of catabolically-released self antigens. The model acknowledges the resulting cost in terms of susceptibility to infection [2,5,6] and accommodates the biologically trivial endpoint that a disintegrative loss of all forms of immune competence must ultimately prevail if wasting disease is permitted to proceed indefinitely.

The tolerance model centers on three anti-inflammatory mediators, namely the glucocorticoids, transforming growth factor(TGF)- $\beta$  and interleukin(IL)-10. This hormonal triad plays a determining role in physiological anti-inflammatory control and the maintenance of self tolerance [7-12], and the constitutive blood levels of these mediators collectively provide a primary, or first tier, of regulatory immune competence positioned to act when an inflammatory stimulus is first encountered. Recently, high constitutive blood levels of each member of this anti-inflammatory triad have been

reported in weanling mouse models of marasmus and incipient kwashiorkor which consistently reproduce the critical features of these pediatric pathologies [4-6,13]. Further, remarkably, the systemic rate of constitutive IL-10 production is at least sustained even into the advanced stages of wasting pathology in the same high-fidelity mouse models of pre-pubescent malnutrition [6].

IL-10 is a particularly important anti-inflammatory mediator that regulates both innate and adaptive immune responses [14,15]. This cytokine is produced by diverse cell types in numerous anatomical locations [15-17], but the cellular sources that sustain its high tissue fluid levels in acute malnutrition are unknown. The objective of this investigation was to determine the influence of acute, pre-pubescent protein and energy deficits on the IL-10-producing potential at major anatomical sites representative of both primary and secondary lymphoid organs and including both systemic and mucosal lymphoid aggregates. The research strategy centered on expression of the IL-10 gene in murine models of weanling malnutrition known to cause elevations in blood levels of IL-10 and known, also, to reproduce the critical features of well-recognized forms of acute pediatric malnutrition including depressed inflammatory immune competence.

## **Materials and Methods**

### *Animals and housing facilities*

Male and female C57BL/6J mice were obtained from an in-house breeding colony derived from animals originally purchased from the Jackson Laboratory (Bar Harbor, ME). Caging and housing conditions were exactly as described previously [18-20], and

this investigation was approved by the Animal Care Committee of the University of Guelph in accordance with the Canadian Council on Animal Care.

#### *Experimental animals, dietary protocols and experimental design*

Eighteen-day-old animals were weaned and acclimated overnight to a complete purified diet described in detail elsewhere [21]. Subsequently, equal numbers of males and females were randomly assigned, at 19 days of age, to one of three experimental groups, namely an age-matched control group consuming the complete purified diet ad libitum, a restricted-intake group consuming the complete purified diet in restricted daily quantities based on calculations that relate ad libitum food intake (g food/g body weight) to chronological age in the weanling mouse [18], or a low-protein group consuming ad libitum a 0.6% crude protein diet (as fed) that was isocaloric with the complete formulation [4,22]. The restricted-intake protocol produces a pathology that reproduces the critical features of pediatric marasmus, whereas the low-protein model closely resembles incipient pediatric kwashiorkor [4,13,18,22-24]. The three dietary groups were maintained on their respective regimens for 14 days, i.e. from 19 through 33 days of age. A sample size of six per dietary group was achieved and the experiment was conducted according to a 2 X 2 design in which the main effects were diet and lymphoid organ (spleen, bone marrow or small intestine).

#### *RNA isolation*

The spleen, bone marrow and small intestine were recovered aseptically and were immediately immersed in ice cold TriPure Isolation Reagent (Roche Diagnostics

Corporation, Indianapolis IN), henceforth designated “TriPure”, for isolation of total RNA. Animals were sacrificed individually in order to minimize the time required to complete the processing of tissue. The spleen was removed intact and immersed in 1 mL TriPure. Bone marrow was flushed from the femur and tibia of both hind limbs by means of a 27½ G needle, and 0.5 mL of RPMI 1640 medium (Flow Laboratories, Mississauga, Canada) containing 10% heat-inactivated fetal bovine serum (Sigma Chemical, St. Louis, MO), 1 mmol/L HEPES (ICN Biomedicals, Aurora, OH),  $10^5$  U/L penicillin and 100 mg/L streptomycin (hereafter designated “medium”) was used for each bone. Excess medium was drained from the resulting marrow plugs which were then immersed in 1 mL TriPure to yield a single pooled sample from each mouse. The small intestine was excised proximally at the pyloric sphincter and distally at the ileocecal junction and was immersed intact in 3 mL TriPure. The intestinal lumen was then cleared of digesta without delay, first by flushing with 10 mL of sterile, ice cold 0.01 M phosphate-buffered saline (PBS, pH 7.3) containing 0.25 mM EDTA to act as a protease inhibitor and secondly by flushing with 10 mL of ice cold TriPure. Following TriPure processing, all tissues were immediately homogenized at room temperature with a Power Gen 125 homogenizer (Fisher Scientific, Ottawa, Ontario, Canada) using a 7 mm x 95 mm saw tooth head. Homogenates were then stored on ice for not more than 10 minutes prior to centrifugation at 12,000 x g for 10 minutes at 4°C to remove cellular debris. Total RNA was isolated from the clear centrifugation supernatant utilizing a RNeasy Mini Kit (Catalogue # 74104, Qiagen Inc, Mississauga, Ontario, Canada) per the manufacturer’s instructions. The procedure included the use of RNase-free DNase to digest any residual

DNA. RNA was quantified by spectrophotometry and absorbance ratios ( $A_{260}/A_{280}$ ) were consistently within the range specified by the manufacturer, i.e. 1.9-2.2.

#### *Assessment of gene expression*

Reverse transcription of 1.5  $\mu$ g of total RNA was achieved using M-MLV Reverse Transcriptase (catalogue #28025013, Invitrogen, Carlsbad CA). Real-time reverse-transcription (RT)-PCR was used to quantify IL-10 mRNA expression and amplification was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). The primer sets for the cytokine, previously used with success [25], were 5'-ggttgccaagccttatcgga-3' (forward) and 5'-acctgctccactgccttgct-3' (reverse). Quantitative RT-PCR was performed using a 7900-HT Real-Time PCR System (Applied Biosystems, Foster City, CA), and relative gene expression was determined using the  $2^{-\Delta\Delta CT}$  method [26] with standardization against  $\beta$ -actin for which the primer sets [25] were 5'-tggaaatcctgtggcatccagtaaac-3' (forward) and 5'-taaaacgcagctcagtaacagtccg-3' (reverse).

#### *Carcass composition*

Animal carcasses were stored at  $-20^{\circ}\text{C}$  to await analysis of dry matter and total lipid content as described previously [13,18].

#### *Statistical analysis*

Statistical analyses were conducted using the SAS system (SAS Institute, Cary, NC) for Windows (version 9.0), and a predetermined upper limit of probability of  $P \leq$

0.05 was applied for statistical significance. Data were subjected to a two-way ANOVA followed, if justified by the level of statistical probability ( $P \leq 0.05$ ), by Tukey's Studentized Range test. If a data set failed to exhibit normal distribution according to each of the four tests applied by the SAS program ( $P \leq 0.05$ ) then a transformation was utilized to bring it into conformity with this basic assumption of parametric testing. Where transformation attempts were unsuccessful, data were subjected to the Kruskal-Wallis test ( $\chi^2$  approximation) followed, if justified by the statistical probability outcome ( $P \leq 0.05$ ), by Wilcoxon two-sample testing.

## **Results**

### *The malnutrition protocols elicited differing weight loss pathologies*

Growth indices for the mice used in this investigation are shown in Table 1. Initial body weights did not differ among dietary groups, whereas final body weights of both malnourished groups were lower than age-matched controls. Food intakes and gains in carcass lean and fat tissue by the age-matched control group were comparable to previous results pertaining to C57BL/6J weanlings given free access to the complete purified diet [4,6,27-29]. Both malnourished groups exhibited low food intakes relative to age-matched controls, including a low level of food intake on a body weight basis. Moreover, both malnourished groups exhibited low levels of carcass fat. However, the restricted-intake protocol induced a greater loss of carcass lipid (and, hence, a greater deficit in carcass energy) than the low-protein protocol as seen previously [4,6,13,28,29]. The performance outcomes of the malnourished groups of this investigation are closely

similar to values reported previously in studies demonstrating reduced inflammatory immune competence in the same experimental systems [18,20,30].

*Expression of the IL-10 gene is high in the spleen in both forms of weanling malnutrition*

The quantity of IL-10 mRNA relative to expression of the  $\beta$ -actin housekeeping gene is shown in Figure 1 for each dietary group and lymphoid site. Viewed in this way, IL-10 gene expression was higher in the spleen of the age-matched control group than in either the small intestine or the bone marrow, and the same outcome emerged in the malnourished groups. Moreover, in the low-protein group, expression of the IL-10 gene in the bone marrow exceeded that in the small intestine. A diet-related difference was apparent in the small intestine in which IL-10 mRNA level was greater in the restricted-intake group than in the low-protein group, although no effect of malnutrition was apparent relative to the age-matched controls. In the spleen, however, the mean expression of IL-10 mRNA exceeded that of the age-matched control group by factors of 2 and 6 in the low-protein and restricted-intake groups, respectively. Splenic IL-10 gene expression of the restricted-intake group also exceeded that of the low-protein group.

Table 1: Performance outcomes and critical composition characteristics of weanling mice after 14-day experimental protocols initiated at 19 days of age<sup>1</sup>

Index	Dietary Group <sup>2</sup>			SEM
	C	LP	R	
Initial body weight (g/mouse)	8.5	8.7	8.6	0.13
Final body weight (g/mouse) <sup>3</sup>	18.6 <sup>X</sup>	6.6 <sup>Y</sup>	6.9 <sup>Z</sup>	----
Food intake (g/mouse · d)	2.9 <sup>X</sup>	1.3 <sup>Y</sup>	0.9 <sup>Y</sup>	0.06
Food intake (g/g body weight · d)	0.2 <sup>X</sup>	0.1 <sup>Y</sup>	0.07 <sup>Z</sup>	0.003
Carcass dry matter (g/100g wet weight)	31.0 <sup>X</sup>	27.8 <sup>Y</sup>	26.3 <sup>Y</sup>	0.004
Carcass lipid (g/100g wet weight) <sup>4</sup>	8.5 <sup>X</sup>	4.1 <sup>Y</sup>	2.4 <sup>Z</sup>	0.05

<sup>1</sup>Mean values. Within a row, values not sharing a superscript letter differ ( $P \leq 0.05$ ) according to Tukey's Studentized Range test except where a different statistical procedure is indicated.

<sup>2</sup>C: group that consumed complete diet ad libitum; LP: group that consumed low-protein diet ad libitum; R: group that consumed complete diet in restricted daily quantities.

<sup>3</sup>Kruskal–Wallis test of Wilcoxon rank sums, which were as follows: C=93.0; LP=26.0; R=52.0.

<sup>4</sup>From ANOVA of natural log-transformed data. Mean values are antilogs of log means.

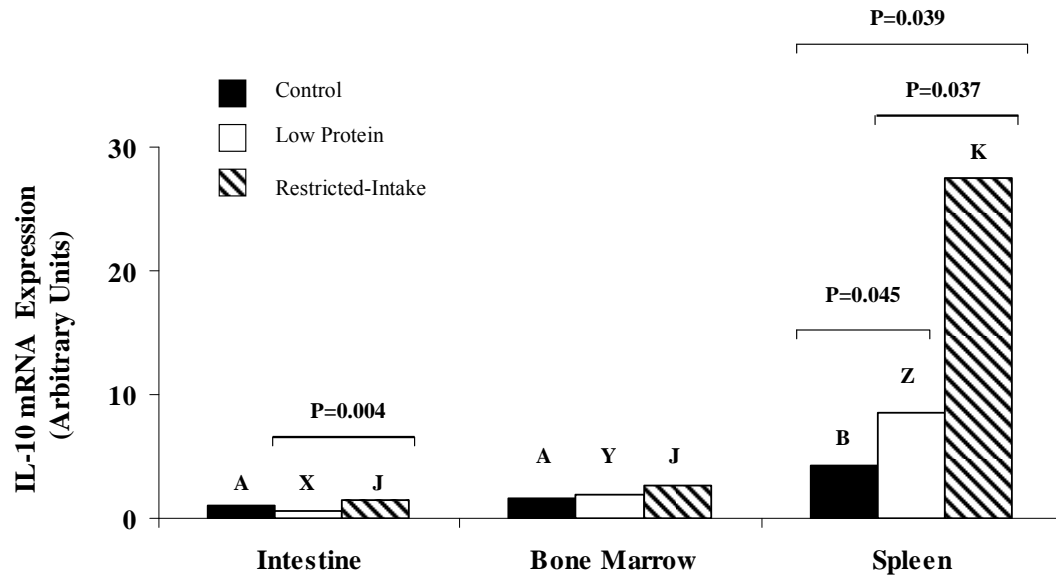


Figure 1. IL-10 mRNA expression in lymphoid organs assessed by real-time reverse-transcription-PCR. C57BL/6J mice initially 19 days old were fed a complete purified diet ad libitum (age-matched controls, black bars), or were given the complete diet in restricted daily quantities (restricted-intake group, striped bars), or were given free access to an isocaloric low-protein diet containing 0.6% crude protein (low-protein group, open bars) for 14 days, 3 males and 3 females in each group. Total RNA was isolated from spleen, bone marrow (combined hind limb femur and tibia) and small intestine. Relative abundance of IL-10 mRNA was determined using the  $2^{-\Delta\Delta CT}$  method [26] with standardization against  $\beta$ -actin. Bars represent means and the data were analyzed by the Kruskal-Wallis test ( $P < 0.0001$ ), followed by Wilcoxon two-sample testing. The Wilcoxon rank sums within each lymphoid site (control, low-protein and restricted-intake

groups, respectively) were as follows: 81, 29, 126 (intestine), 137, 138, 184 (bone marrow) and 220, 268, 304 (spleen). Within dietary groups, bars not sharing an upper case letter denote differences between anatomical sites ( $P \leq 0.05$ ) wherein letter assignments are as follows: age-matched controls (AB), low-protein (XYZ) and restricted-intake (JK). Statistically significant differences ( $P \leq 0.05$ ) between dietary groups within organ sites are identified by showing the P value.

## **Discussion**

The present investigation centers attention on the spleen as a particularly important site of constitutive IL-10 mRNA expression in the healthy adolescent mouse and reveals that the physiological splenic anti-inflammatory potential is exaggerated in two models of acute weaning protein and energy deficit. Thus, the spleen emerges as an immunological site that is likely to play a particularly significant role in support of the high levels of IL-10 that are sustained into the advanced stages of acute weaning malnutrition [4,6]. These findings were apparent in two metabolically distinct murine models of acute pre-pubescent malnutrition which consistently depress adaptive inflammatory immune competence [18,20,30] and which mimic the well-defined pediatric pathologies of marasmus via the restricted-intake model and incipient kwashiorkor via the low-protein model [4,18,19,31]. Further, IL-10 gene expression was at least sustained into the advanced stages of nutritional pathology in two major lymphoid sites in addition to the spleen. Overall, this investigation lends new support to the tolerance model of anti-inflammatory immune regulation in pre-pubescent protein and energy deficit.

The intestine is the largest mucosal immunological organ in the mammalian body [32] is designed to provide a non-inflammatory type of defence based on IgA-class immunoglobulins which function as blocking antibodies that inhibit the absorption of antigens from the intestinal lumen [33]. In the capacity of a secondary lymphoid organ, the bone marrow is comparable in size to the intestine [32] and is, likewise, a particularly important source of IgA-class immunoglobulins [34]. The present investigation, therefore, reveals that the potential to produce a key anti-inflammatory cytokine is

sustained into the advanced stages of weanling malnutrition in two types of secondary lymphoid organs that are designed to provide non-inflammatory immunological defence. In this connection, it is of interest that IL-10 promotes IgA class-switching in vitro [35], and an elevated blood level of IgA is common in the advanced stages of acute pre-pubescent malnutrition [3,36] including the weanling low-protein model used herein [37]. By contrast, the spleen is the largest of all lymphoid organs [32] and characteristically supports inflammatory responses. Moreover, a dominant role is emerging for the spleen in controlling physiological lymphocyte recirculation dynamics [38]. It is interesting, therefore, that the potential for IL-10-centered anti-inflammatory regulation was elevated in this site despite an increasingly limited supply of substrates and energy as wasting disease progressed. In view of the size and immunobiology of the spleen, the present investigation points to this organ as a significant contributor both to physiological IL-10 production and in supporting the high tissue fluid levels of IL-10 reported elsewhere [4,6] in weanling malnutrition pathology. The splenic cellular sources of IL-10 in acute malnutrition remain to be identified.

The metabolic dissimilarities between mice subjected to the low-protein and restricted-intake protocols, and their respective similarity to the human pathologies of kwashiorkor and marasmus, have been noted elsewhere [19,28,31]. Briefly, the low-protein diet produces weight loss consequent to dietary imbalance, and weanling mice consuming this diet develop fatty liver and edema, the hallmark signs of kwashiorkor, after four weeks or more of wasting disease. When imposed for the periods of time reported in this investigation, therefore, the low-protein protocol produces an incipient kwashiorkor. In contrast, the restricted-intake protocol induces weight loss apart from

dietary imbalance. This protocol stimulates the extreme mobilization of fat reserves that would be expected in a model of marasmus but fails to produce the hepatic pathology seen in mice subjected to the low-protein protocol. Further, the restricted-intake system produces a vigorously gluconeogenic animal whereas the low-protein diet, with its high level of digestible carbohydrate, has the opposite metabolic influence [31,39]. Despite their qualitative metabolic differences, these forms of experimental malnutrition have consistently exhibited immunological (including cytokine) characteristics that differ only quantitatively [2,4,19,22,24,28,29]. The findings reported herein appear consistent with this broadly based conclusion. A search for biologically substantive immunological distinctions is of interest among forms of acute malnutrition but is beyond the design and scope of this investigation.

The expression of mRNA and its corresponding protein do not necessarily correlate. However, where diet-related differences emerged in the expression of the IL-10 gene in the present investigation, these differences mirrored the relative systemic expression of IL-10 protein reported elsewhere [6] in the same experimental models. Moreover, the parallel between gene expression (this investigation) and protein expression [6] was precise in the spleen which proved to be a site of particularly vigorous constitutive IL-10 mRNA expression independently of protein and energy status. Significantly, IL-10 gene expression was at least sustained, despite limited supplies of nitrogen and energy, even into the advanced stages of weight loss in each lymphoid site examined. Overall, the findings of this investigation indicate that the high tissue fluid levels of IL-10 in acute malnutrition reflect, at least in part, new cytokine production rather than simply a decreased rate of cytokine turnover. In view of the physiological

role of IL-10 as a potent anti-inflammatory mediator [15,40], these findings lend new support to the proposition that the characteristic depression in cell-mediated immune capability in acute pre-pubescent malnutrition reflects a regulated, non-inflammatory form of immune competence [2-6]. This emerging model is the antithesis of the classic and prevalent model of chaotic malnutrition-induced immunological disintegration and centers on minimizing the risk of inflammatory autoimmune disease in the face of catabolically-released self antigens. The present investigation provides the first mechanistic support for this important new hypothesis.

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## Chapter Ten

### Summary and Perspectives

The underlying causes of acute malnutrition-associated inflammatory immune depression remain unknown. However, when considered as a whole, the objectives of this thesis were centred on two potential contributing factors supporting this phenomenon, namely the maintenance of the anti-inflammatory and tolerogenic form of immune competence and the propensity toward type-2 polarization of immune responses in acute pre-pubescent malnutrition. The investigations in this thesis stem from and extend the previously documented hypothesis that wasting-associated immunobiology must be understood in the context of a hormonally-mediated adaptive response to acute deficits of protein and/or energy [1-3]. Previous reports determined that, in the advanced stages of acute malnutrition, the blood and tissue fluid levels of the glucocorticoids (GC) [4], interleukin(IL)-10 and transforming growth factor(TGF)- $\beta$  [5] were elevated, whereas the concentration of the inflammatory cytokine, interferon(IFN)- $\gamma$ , was depressed [6]. Together, these late hormonal changes indicated the likelihood of a propensity toward a type-2 polarization of immune responses and the maintenance of an anti-inflammatory form of immune competence in the most advanced stages of weight loss pathology. The studies presented in this thesis were designed with a view to the following in acute pre-pubescent malnutrition: i) to provide depth in the understanding of the broad hormonal response in PEM, ii) to provide a basis that supports the potential implication of the anti-inflammatory hormonal triad in the initiation of inflammatory immune depression and iii) to search for direct evidence of a type-2 polarization.

In response to acute deficits of protein and/or energy, an early rise in the blood and tissue fluid levels was determined for each of the hormones that comprise the anti-inflammatory triad, the GC [7], TGF- $\beta$  [8] and IL-10 [9]. Collectively, these findings add a new perspective with respect to the early hormonal response to wasting deficits of protein and/or energy. The anti-inflammatory hormonal triad (GC, TGF- $\beta$  and IL-10) appears to comprise the basis that supports the preservation of the non-inflammatory form of immune competence in wasting pathology, and is positioned temporally to play both an initiating and a sustaining role in inflammatory cell-mediated immune depression. The ability to function as an inter-related triad, exerting an overall anti-inflammatory effect in wasting malnutrition is linked to their common immunological functions. Each member of the hormonal triad has been shown to positively modulate the production of the others, and thus the potential of synergistic relationships between and among the GC, TGF- $\beta$  and IL-10 remains a compelling possibility in wasting malnutrition [10-20]. These hormones have been identified as the key mediators that function to prime the cellular microenvironment in a manner that promotes immunological tolerance by actively suppressing inflammatory cell-mediated immune responses, as depicted in Figure 1 [21-28]. The potential influences of the hormonal triad on the cellular microenvironment stem from their redundant tolerogenic influences on both APCs and T cells. APC maturation blockade promotes the development of tolerogenic APCs [22,29-32]. In this context, it is interesting that, in advanced weanling protein and energy deficit, a large proportion of splenic DCs express a tolerogenic surface phenotype [33]. Additionally, in acute pre-pubescent malnutrition, an overabundance of T cells exhibiting a quiescent or naïve phenotype is apparent [34-36]. Thus, tolerogenic

APCs may influence naïve T cells to differentiate to become IL-10/TGF- $\beta$ -producing regulatory T cells (Tr1) or TGF- $\beta$ -producing regulatory T cells (Th3) which, in turn, actively suppress inflammatory immune responses and support high tissue fluid levels of these cytokines [21,27,37]. Concomitantly, the permissive influence of the tolerogenic hormonal triad must be noted with respect to the development of type-2 polarization within the effector T cell compartment [38], and thus, may provide an alternative end-stage differentiation fate for naïve T cells. The combined effect of these proposed simultaneous mechanisms (enhanced immunological tolerance and type-2 polarization) is a depressive influence on the capacity to generate an inflammatory cell-mediated immune response. This model encompasses the classic observation in acute malnutrition of depressed inflammatory cell-mediated immune responses and unpredictably affected humoral responses [1,39,40] while providing a basis for this phenomenon that can be explained as part of a larger physiological response to acute malnutrition. This extends the idea of physiological tolerogenic control to include an active influence throughout the duration of weight loss in response to acute pre-pubescent malnutrition.

Some further evidence can be cited in relation to the proposed tolerogenic character of the cellular microenvironment in acute pre-pubescent malnutrition. Specifically, FOXP3 mRNA expression is sustained in major lymphoid organs in acute weanling malnutrition (Figure 2). FOXP3 is a transcription factor that is uniquely expressed by regulatory T cells, and whose expression can be induced or supported in the periphery by the triad of GC, TGF- $\beta$  and IL-10 [23,41-43]. Further, among anatomical sites assessed, independent of dietary group, FOXP3 expression was highest in the spleen, a similar outcome to that of IL-10 mRNA expression (Chapter 9). Collectively

these data point to the spleen as a likely anatomical site of tolerogenic regulation in wasting malnutrition. Interestingly, some hints that the spleen has a tolerogenic role under physiological conditions can be found in previous studies of splenectomized experimental animals [44]. It is unknown if the elevated levels of the anti-inflammatory and tolerogenic hormones in acute malnutrition represent increased production, reduced catabolism/turnover, or both. New cytokine synthesis remains a compelling explanation and evidence supporting this notion emerged with respect to IL-10. In the acutely malnourished, IL-10 mRNA expression, and hence, IL-10-producing potential, is sustained in secondary lymphoid organs but profoundly elevated in the spleen (Chapter 9). Further, systemic constitutive IL-10 protein production is at least sustained in the acutely malnourished weanling mouse regardless of the stage of weight loss [9]. These findings extend the proposition that malnutrition-associated changes in immune function represent an actively regulated process, despite limitations in energy and nitrogen [2].

In light of the early and sustained rise in anti-inflammatory hormones and the overlapping influences of these mediators on the maintenance of peripheral self-tolerance, a new tolerance-centered model emerges that encompasses and extends the original hormonal hypothesis of malnutrition-associated immune depression. Thus the hormonal triad of GC, TGF- $\beta$  and IL-10 perpetuates an anti-inflammatory cellular environment that re-directs immune responses away from the capacity to generate a classical type-1 inflammatory cell-mediated response but is permissive of the type-2 polarization of immune responses that is often preserved in advanced wasting pathology [1,38]. These phenomena appear to be actively regulated despite limitations in nitrogen and energy [9,38]. This interpretation challenges the prevailing notion that immune

depression in wasting malnutrition is the result of a chaotic disintegrative process whereby immunological capabilities representing 15% of the basal metabolic rate [45] are sacrificed in order to sustain more essential physiological processes. At the same time, the model acknowledges that as weight loss progresses, immunological capacities must be subject to disintegration, and the findings reported herein (Chapter 7 and 8) suggest that the second-tier type of anti-inflammatory competence is vulnerable first.

The model depicted in Figure 3 is a modified version of the classic malnutrition-infection cycle [46]. The figure identifies the adaptive benefit conferred by an actively regulated anti-inflammatory and tolerogenic form of immune competence in wasting malnutrition, i.e. the active suppression of inflammatory immune responses generated against catabolically released self-antigens. In this model, by addressing the more immediate threat, which is the development of autoimmune reactions, an active depression in classical inflammatory cell-mediated immune competence results as a by-product that, in turn, increases susceptibility to opportunistic infection. From a clinical perspective, this revised model is significant because a regulated phenomenon must be susceptible to management intervention.

An interesting perspective emerges from the outcomes in this thesis when considered within an energy balance spectrum. On one end, in its most severe wasting forms, is acute protein energy malnutrition, an obvious state of negative energy balance. At the opposing end, in a state of positive energy balance, is the obese condition. Supported by the findings in this thesis, acute protein and energy deficit may be characterized by an anti-inflammatory and tolerogenic form of immune competence. Conversely, obesity is associated with a low-grade chronic inflammatory state [46] and

an increasing amount of evidence suggests that obesity increases the risk of autoimmune disease development [48]. Evidence of type one polarization exists in the obese condition [49,50]. Further, the high leptin concentrations associated with obesity [48] impair regulatory T cell activation and proliferation [51] and regulatory T cell populations are reduced in size in the obese [51,52]. Thus chronic positive energy balance appears to be associated with tolerogenic dysfunction, inflammatory pathology and autoimmune susceptibility, whereas under-nutrition is associated with depressed inflammatory immune competence, preserved anti-inflammatory and tolerogenic forms of immunity and thus, decreased susceptibility to autoimmunity.

The opposing physiological responses to the extremes of energy balance appear to result in opposing tolerogenic capabilities. Therefore, understanding the pathophysiology of these conditions from a tolerogenic standpoint may provide insight into the underlying immunological changes that ultimately adversely impact immune function in these pathologies.

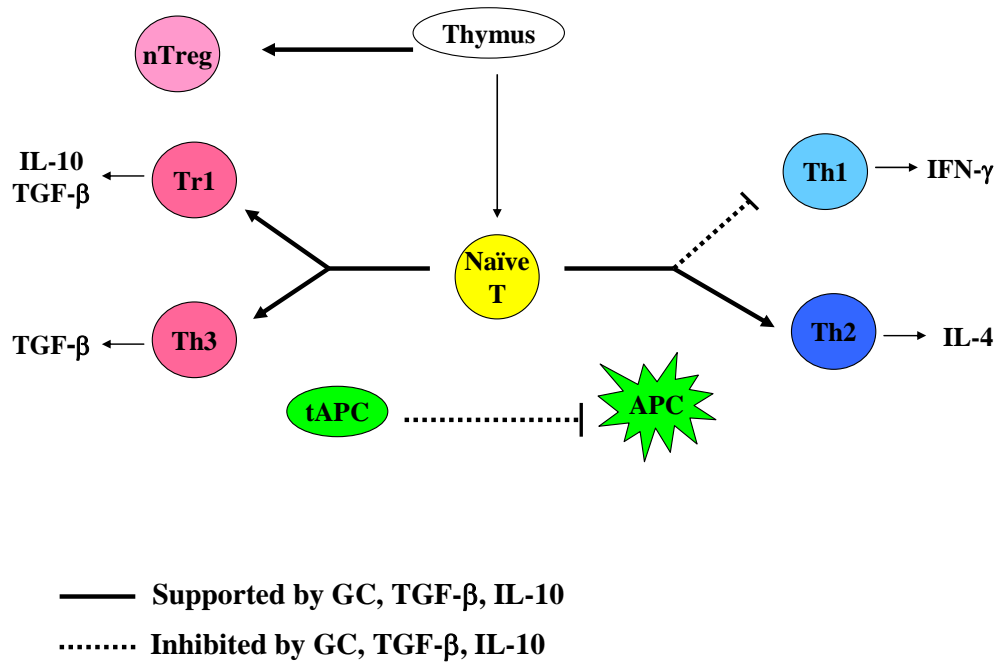


Figure 1. How the tolerogenic triad may influence the cellular microenvironment in acute pre-pubescent malnutrition. These influences include the maturational blockade of APCs, the development of inducible regulatory T cells and the propensity toward type-2 polarization of immune responses. Abbreviations: tolerogenic antigen-presenting cell (tAPC), naturally occurring regulatory T cell released from the thymus (nTreg), type-1 inducible regulatory T cell (Tr1), type-3 inducible regulatory T cell (Th3).

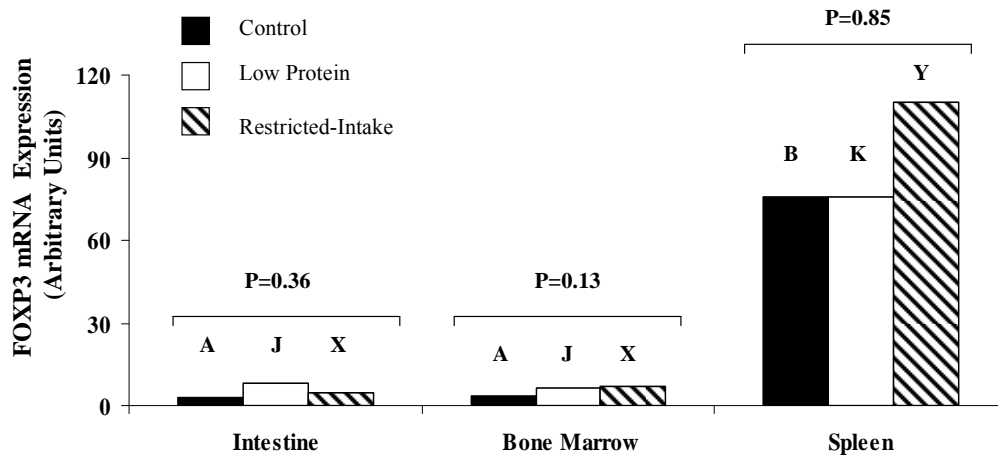


Figure 2. FOXP3 mRNA expression in secondary lymphoid organs assessed by real time reverse-transcription-PCR. C57BL/6J mice initially 19 days old were fed a complete purified diet ad libitum (age-matched controls, black bars), or were given the complete diet in restricted daily quantities (restricted-intake group, striped bars), or were given free access to an isocaloric low-protein diet containing 0.6% crude protein (low-protein group, open bars) for 14 days, n=6/dietary group. Total RNA was isolated from spleen, bone marrow (combined hind limb femur and tibia) and small intestine. Relative abundance of FOXP3 mRNA was determined by quantitative reverse-transcribed (RT)-PCR using the  $2^{-\Delta\Delta C_t}$  method with standardization against  $\beta$ -actin. Bars represent means

and were analyzed by the Kruskal-Wallis test ( $P < 0.0001$ ), followed by Wilcoxon two-sample testing. The Wilcoxon rank sums within each lymphoid site (control, low-protein and restricted-intake groups, respectively) were as follows: 76, 89, 120 (intestine), 88, 145, 154 (bone marrow) and 264, 275, 275 (spleen). Within dietary groups, bars not sharing an upper case letter denote differences between anatomical sites ( $P \leq 0.05$ ) wherein letter assignments are as follows: age-matched controls (*AB*), low-protein (*JK*) and restricted-intake (*XY*). No differences were apparent among dietary groups within organ sites ( $P \geq 0.05$ ).

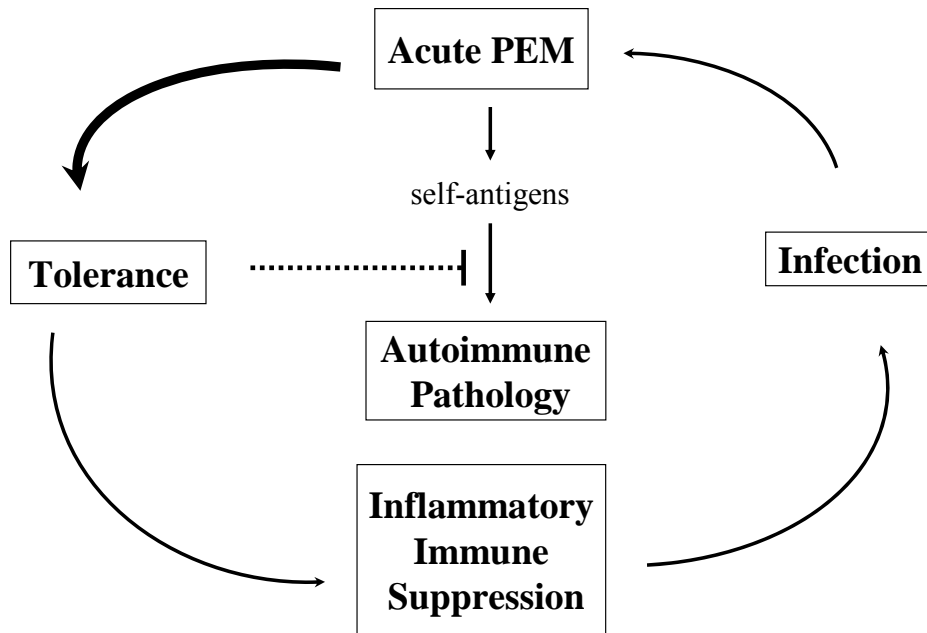


Figure 3. The modified version of the classic malnutrition-infection cycle in acute pre-pubescent malnutrition. Bold arrow represents the sustained constitutive anti-inflammatory and tolerogenic form of immune competence in acute malnutrition, the hormonal triad comprised of GC, TGF- $\beta$  and IL-10

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## Appendix 1: Composition of Purified Diets<sup>1</sup>

<b>Ingredient</b>	<b>Complete (g/kg)</b>	<b>Low Protein (g/kg)</b>
Spray-Dried Egg White <sup>2</sup>	245	1.5
Cornstarch <sup>3</sup>	290	533.5
Glucose <sup>4</sup>	290	290
Cellulose <sup>5</sup>	50	50
Corn Oil <sup>6</sup>	75	75
Vitamin-Mineral Supplement <sup>7</sup>	50	50

<sup>1</sup>A typical proximate analysis of the complete diet is as follows: 92.3% dry matter, 18.8% crude protein, 8.1% ether extract, 2.6% ash, 3.1% crude fibre and 17.0kJ/g gross energy [Woods and Woodward, 1991].

<sup>2</sup>80% Crude protein, U.S. Biochemical Corp., Cleveland, OH.

<sup>3</sup>St. Lawrence Starch Company, Ltd., Port Credit, Ont.

<sup>4</sup>Cerelose, 2001, CPC International, Englewood Cliffs, N.J.

<sup>5</sup>Celufil, non-nutritive bulk, U.S. Biochemical Corp.

<sup>6</sup>Contained sufficient supplemented fat-soluble vitamins to provide per kilogram diet: 1000 IU cholecalciferol and 50 IU all-rac- $\alpha$ -tocopherol acetate.

<sup>7</sup>The supplement supplied the following levels of nutrients per kilogram diet: 4000 IU retinyl acetate; 0.8 mg menadione sodium bisulfate; 4 mg biotin; 1mg folic acid; 15 mg niacin; 12 mg D-calcium pantothenate; 10 mg riboflavin; 8 mg thiamine HCL; 9 mg pyridoxine HCL; 0.05 mg cyanocobalamin; 1.2 g choline chloride; 2.5 g sodium chloride; 27.8g dicalcium phosphate (20% calcium, 18% phosphate); 0.9 g magnesium oxide (60% magnesium); 10.7 g potassium sulphate (45% potassium); 21.6 mg cupric sulphate (25% copper); 174 mg ferrous sulphate; 21 mg manganous sulphate (33% manganese); 0.3 mg potassium iodide (75% iodide); 0.4 mg sodium selenite (30% selenium); 23 mg zinc carbonate (52% zinc); 5.3 mg chromium chloride (33% chromium). The supplement was brought to the appropriate weight with glucose.