Available online at www.sciencedirect.com
ScienceDirect
Journal of Nutritional Biochemistry 21 (2010) 504–511
Journal of Nutritional Biochemistry

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

Tessa A.M. Steevels, Lyn M. Hillyer, Jennifer M. Monk, Megan E. Fisher, Bill D. Woodward*

Department of Human Health and Nutritional Sciences, University of Guelph, Ontario, Canada N1G 2W1

Received 14 July 2008; received in revised form 21 January 2009; accepted 16 February 2009

Abstract

Our 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 prepubescent malnutrition. Weanling C57BL/6J 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)-γ 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-γ 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-γ (P=.002) but more IL-4 (P=.05) by T cells from the restricted-intake group relative to the age-matched control group. Similarly, in vivo stimulation generated low serum levels of antibody-captured IFN-γ in the restricted-intake group vis-à-vis the age-matched control group (P=.01), while the IL-4 response was sustained (P=.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-γ were low after anti-CD3 challenge relative to the age-matched control group (P=.02), while levels of captured IL-4 remained unaffected (P=.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.

Keywords: Acute protein–energy malnutrition; Cytokine; Interferon-gamma; Interleukin-4; Mouse; T cell

1. Introduction

Acute (i.e., wasting) prepubescent deficits of protein and/or energy in their most severe forms — marasmus and kwashiorkor — are associated with up to 2 million largely infection-related deaths annually among children under the age of 5 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 prepubescent 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 immunoglobulin G (IgG) antibody that support this type of response [6], which is designed for defense against intracellular pathogens [5,7]. By contrast, Type 2 cytokines promote the production of antibodies whose main function is to provide protection in the extracellular space [5,7].

Interferon (IFN)-γ 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, IL-5, IL-6, IL-10 and IL-13; IL-4 is regarded as the primary polarizing cytokine of this group [5,7]. Both IFN-γ and IL-4 arise from a variety of cellular sources, including T cells [4,7]. To acquire the capacity to produce cytokines that reflect the end-stage polarity of an adaptive immune response, such as IFN-γ and IL-4, T cells must achieve effector/memory status [8]. Naive T cells require 24–48 h of stimulation to commit to effector

* Corresponding author. Tel.: +1 519 824 4120x53741; fax: +1 519 763 5902.
E-mail address: wwoodwar@uoguelph.ca (B.D. Woodward).

0955-2863/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.jnutbio.2009.02.007

polarization in vitro [8,9] and can commit to effector differentiation only if subjected to simultaneous stimuli through the T-cell receptor and 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-γ 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 h, such as those used in the cited studies, permit activation of naive T cells [9]. Moreover, the cited studies are not directly relevant to the prepubescent stage of life. Therefore, the first objective of this investigation was to determine the influence of metabolically distinct forms of acute prepubescent malnutrition, mimicking marasmus and incipient kwashiorkor, on the polarity of the effector/memory T-cell compartment as indicated by the production of IFN-γ 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 recorded depressed production of IFN-γ mRNA in vivo in response to experimental tuberculosis [15]. However, the design of that 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 as 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-γ, IL-2, IL-4, IL-6 and IL-10 [17], and permits simultaneous assessment of more than one cytokine, as has been demonstrated for IFN-γ 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-γ 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, USA). Caging and housing conditions were exactly as described previously [18–23], and the investigation was approved by the University of Guelph Animal Care Committee 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 a 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 fiber and 17.0 kJ/g of 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, USA) of the complete diet with an equal weight of cornstarch (ICN Biomedicals, Aurora, OH, USA). 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 in which all groups except the zero-time control group were maintained on their respective regimens for 14 days (i.e., from 19 through 33 days of age) were performed. 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; 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 10 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. Pooled samples were required for mice subjected to the low-protein and restricted-intake groups in order to obtain sufficient numbers of cells. 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 2×2 design in which diet and in vivo polyclonal T-cell stimulation (using anti-mouse CD3) were the main effects. Eight mice (4 males and 4 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 in the case of the zero-time controls, 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°C to await analysis.

A small supplementary experiment in which 19-day-old C57BL/6J mice were given free access to either the complete diet or the low-protein diet for 26 days was performed. Six mice (three males and three females) were assigned to each dietary group, and the experiment centered on IFN-γ and IL-4 production in vivo in response to stimulation with anti-CD3. The outcome of the experiment was therefore assessed by means of a blood sample taken at the end of the 26-day experimental period. Animal carcasses were stored at −20°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_2 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, Ontario, Canada) containing 10% heat-inactivated fetal bovine serum (Sigma Chemical, St. Louis, MO, USA), 1 mmol/L Hepes (ICN Biomedicals), 10^5 U/L of penicillin and 100 mg/L of 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, Franklin Lakes, NJ, USA) were coated overnight at 4°C with 200 μl of 0.01 M phosphate-buffered saline (PBS; pH 7.3) containing 5 μg/ml of anti-CD3 (clone 145-2C11; Cedarlane Laboratories, Hornby, Ontario, Canada). After coating, plates were washed twice with PBS and each well received 2×10^5 viable mononuclear cells in 190 μl of 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). Negative control cultures were produced using wells not coated with anti-CD3, and the cells were cultured in fluids comprising 190 μl of complete medium together with 10 μl of PBS. All cultures were incubated at 37°C for 24 h. After incubation, plates were centrifuged for 1 min at 200*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-γ (BD Biosciences, Mississauga, Ontario, Canada) were applied to samples as described by the manufacturer. Outcomes were quantified by optical density using a VMax kinetic plate reader (Molecular Devices, Menlo Park, CA, USA) 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 the percentage of $CD3^+$ cells in mononuclear cell suspensions from spleen and lymph nodes

Analyses were performed using a Becton-Dickinson FACSCalibur flow cytometer equipped with BD CellQuest 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 10^4 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 CD3ε (145-2C11, hamster IgG; eBiosciences, San Diego, CA, USA) at a concentration of 0.2 μg per 250×10^3 viable cells. Negative control samples were stained with biotin-conjugated hamster IgG (Cedarlane Laboratories) followed by phycoerythrin-conjugated streptavidin (Cedarlane Laboratories) at concentrations of 0.2 and 0.15 μg, respectively, per 250×10^3 viable cells. Cells were incubated with staining reagents in the dark on ice for 40 min and 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-γ

The assays were conducted basically as described before [16,17]. Briefly, each mouse received, by intraperitoneal injection, 10 μg of biotin-conjugated anti-mouse IL-4 (BVD6-24G2, rat IgG1; eBiosciences) together with 10 μg of biotin-conjugated anti-mouse IFN-γ (R4-6A2, rat IgG1; eBiosciences). The antibodies were delivered in 125 μl of endotoxin-free physiological saline (henceforth, "saline") and given to the animals at the end of the 14-day experimental period; in the case of the zero-time control group, they were given at 19 days of age immediately following the 24-h acclimation period. Half of the mice received 10 μg of anti-mouse CD3 (145-2C11, Armenian hamster IgG; BD Biosciences, San Diego, CA, USA) 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-γ (BD Biosciences, San Diego, CA, USA). Finally, isotype control animals (n=2 per group) were included, and each animal received 10 μg of biotin-conjugated rat IgG1 (KLH/G1-2-2; Cedarlane Laboratories) by intraperitoneal injection in 125-μl saline. Controls for mice stimulated with anti-CD3 (n=2 per group) received 10 μg of Armenian hamster IgG (G235-2356; BD Biosciences, San Diego, CA, USA) in the same saline-based injection.

2.8. Blood collection

After measurement of body weight, mice were anesthetized with CO_2 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 min. 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 analysis

The predetermined upper limit of probability for statistical significance throughout this investigation was $P\leq.05$, 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 analysis of variance (ANOVA) or a two-way ANOVA; in the latter case, diet and T-cell stimulus served as main effects. If justified by the resulting probability value (i.e., $P\leq.05$), multiple-group analyses were extended by application of the 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 preplanned 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 (χ^2 approximation), followed, if justified by the statistical probability outcome ($P\leq.05$), by Wilcoxon two-sample testing. Nonparametric testing of data sets involving only two groups was accomplished by means of Wilcoxon two-sample comparisons.

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

Index	Dietary group[1]				S.E.M.
	B	C	LP	R	
Experiment 1: Cytokine production in vitro					
Initial body weight (g/mouse)	8.3	8.3	8.6	8.1	0.25
Final body weight (g/mouse)[2]	–	16.1^{A}	6.4^{B}	6.3^{B}	0.03
Food intake (g/mouse×14 days)[2]	–	46.4^{A}	18.6^{B}	11.7^{C}	0.03
Food intake (g/g body weight×days)[2]	–	0.27^{A}	0.18^{B}	0.12^{C}	0.04
Carcass dry matter (g/100 g of wet weight)	29.7^{AB}	31.2^{A}	27.7^{BC}	26.4^{C}	0.57
Carcass lipid (g/100 g of wet weight)[3]	8.6^{A}	8.5^{A}	4.8^{B}	2.2^{C}	–
Percentage of $CD3^+$ mononuclear cells in spleen and nodes[4]	18.9^{C}	29.9^{BC}	44.7^{A}	39.0^{AB}	3.36
Experiment 2: Cytokine production in vivo					
Initial body weight (g/mouse)[5]	8.3	8.6	8.8	8.6	2.97
Final body weight (g/mouse)[2]	–	17.9^{A}	6.7^{B}	6.4^{B}	0.02
Food intake (g/mouse×14 days)[2]	—	65.1^{A}	21.2^{B}	11.5^{C}	0.03
Food intake (g/g body weight×days)[2]	–	0.26^{A}	0.13^{B}	0.07^{C}	0.03
Carcass dry matter (g/100 g of wet weight)[6]	28.1^{B}	32.1^{A}	26.5^{C}	26.3^{C}	–
Carcass lipid (g/100 g of wet weight)[2]	8.0^{A}	9.2^{A}	4.8^{B}	2.7^{C}	0.05

Mean values are shown. Within a row, values not sharing a superscript letter differ ($P\leq.05$) according to the Tukey's Studentized Range test or the Kruskal–Wallis procedure followed by Wilcoxon two-sample comparisons.

[1] B=zero-time control, 19 days 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.

[2] From ANOVA of natural log-transformed data. Mean values are antilogs of log means.

[3] Kruskal–Wallis test of Wilcoxon rank sums, which were as follows: B=247.0; C=243.0; LP=160.5; and R=90.5.

[4] Percentage of cells expressing CD3ε in the total mononuclear cell population identified by forward-angle light scatter in the flow cytometer.

[5] From ANOVA of square-transformed data. Mean values are square roots of squared means.

[6] Kruskal–Wallis test of Wilcoxon rank sums, which were as follows: C=880.0; B=535.5; LP=379.5; and R=285.0.

3. Results

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

Growth indices, shown in Table 1, 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 with 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 with 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 that 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

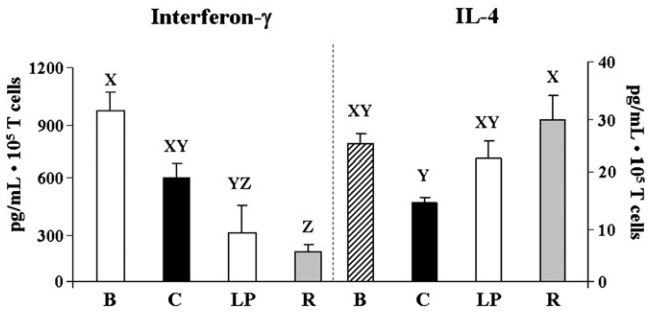

Fig. 1. Concentrations of IFN-γ and IL-4 found in cultures of mononuclear cells from the spleen and lymph nodes after 24 h in vitro. Each culture included 2×10^5 viable cells, and polyclonal stimulation was achieved with plate-bound anti-CD3 (145-2C11, 5 μg/ml) and, in some cultures, soluble anti-CD28 (37.51.1, 20 μg/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-day 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-day-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 S.E.M. 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±anti-CD28). Means are antilogs of log-transformed means, and diet main effect P values were .002 and .05 for IFN-γ and IL-4, respectively. Bars not sharing an upper case letter differ ($P\leq.05$) according to the Tukey's Studentized Range test, and pooled S.E.M. values were 0.336 and 0.195 for IFN-γ and IL-4, respectively. The diet effect was independent of stimulus (interaction $P=.95$ and .65 for IFN-γ and IL-4, respectively). The stimulus main effect (not shown) was statistically significant for both cytokines ($P<.0001$), but anti-CD28 stimulation elicited no further production of either IFN-γ or IL-4 beyond the response to anti-CD3 alone ($P>.05$, the Tukey's Studentized Range test).

energy) than the low-protein protocol. Moreover, the food intakes of the malnourished groups (low relative to the intake of the age-matched control group) 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-γ 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 concentrations of IL-4 and IFN-γ detected in culture fluids of the mononuclear cell suspensions relative to the cytokine levels supported by cultures not exposed to stimulation in vitro ($P<.0001$ 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 ($P=.10$ and .64 for IFN-γ and IL-4, respectively, according to the Wilcoxon two-sample test). The concentrations of IL-4 and IFN-γ did not differ among dietary groups in culture fluids of mononuclear cells that were not subjected to polyclonal stimulation (results not shown; $P=.21$ and .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 $CD3\varepsilon^+$ 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 $CD3^+$ 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 control group compared with zero-time control group). 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 $CD3^+$ cell basis, the mononuclear cells recovered from the restricted-intake group supported a higher concentration of IL-4 but a lower concentration of IFN-γ 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-γ found in culture fluids. No ontogeny was apparent in this index for either cytokine (age-matched control group compared with zero-time control group). 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; $P>.05$ for each cytokine according to the Tukey's Studentized Range test).

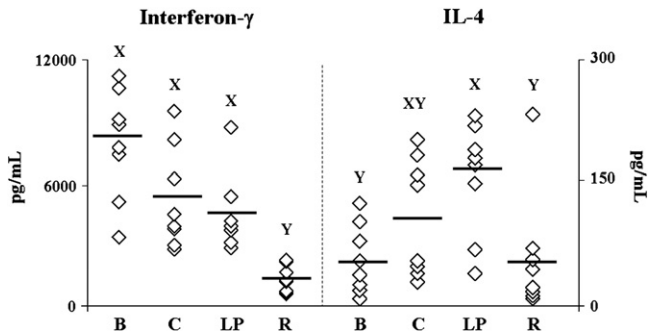

Fig. 2. Concentrations of IFN-γ and IL-4 complexed with their respective biotin-conjugated anti-cytokine antibody (R4-6A2 and BVD6-24G2) in the serum of C57BL/6J mice 4 h after intraperitoneal injection of the biotin-tagged probes together with 10 μg/mouse of anti-CD3 (145-2C11). Malnourished mice were 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-day experimental period. Age-matched controls (group C) were permitted free access to the purified complete diet for the same length of time, and 19-day-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 ($P\leq.05$) according to Wilcoxon two-sample comparisons. Kruskal–Wallis rank sums were 191, 131, 139 and 67 (groups B, C, LP and R, respectively; $P=.01$) for the data pertaining to the serum IFN-γ complex and were 110, 135, 190 and 93 (groups B, C, LP and R, respectively; $P=.05$) for the data pertaining to the serum IL-4 complex.

Table 2
Performance outcomes, including critical composition characteristics, of weanling mice at 19 days of age or after the 26-day low-protein dietary protocol initiated at 19 days of age

Index	Dietary group[1]	
	C	LP
Initial body weight (g/mouse)[2]	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[A]
Food intake (g/mouse×26 days)	79.0±10.15	29.8±4.53[A]
Food intake (g/g body weight×days)	0.21±0.033	0.16±0.026[A]
Carcass dry matter (g/100 g of wet weight)[3]	34.1 (31.8–36.4)	26.8 (19.8–29.8)[A]
Carcass lipid (g/100 g of wet weight)	10.4±1.01	3.7±0.50[A]

Mean values (range or ±S.D.) are shown. Within a row, the superscript letter "A" signifies statistical difference ($P \leq .05$) from the age-matched control group (designated as "C") according to two-tailed Student's *t* test or to Wilcoxon two-sample comparison.
[1] C=group that consumed complete diet ad libitum; LP=group that consumed low-protein diet ad libitum.
[2] Wilcoxon rank sums were 154.0 and 146.0 for groups C and LP, respectively.
[3] Wilcoxon rank sums were 222.0 and 78.0 for groups C and LP, respectively.

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 difference 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 group) in the blood serum concentration of biotin-conjugated anti-cytokine complexes of either IL-4 ($P=.56$) or IFN-γ ($P=.12$) that accumulated in the 4-h test period. Mean values of the four groups of mice (not shown) ranged from 0 to 4 pg/ml and from 0 to 47 pg/ml for IL-4 and IFN-γ, respectively, when corrected for negative (isotype) control values that averaged 86 pg/ml (IL-4) and 57 pg/ml (IFN-γ). Stimulation with anti-CD3 increased the 4-h accumulation of both cytokine complexes in the blood serum independently of dietary group ($P<.0001$, 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-γ). No ontogeny was apparent in this index for either cytokine (zero-time control group compared with age-matched control group). The restricted-intake group exhibited a low level of IFN-γ accumulation in the serum relative to both the age-matched control group 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 4-h accumulation of either cytokine complex in the blood serum but exhibited an elevated serum concentration of IL-4 complex relative to the zero-time control group. 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 in which the low-protein diet was fed to weanling C57BL/6J mice, initially 19 days old, was performed 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-γ complex in the malnourished group relative to the age-matched control group 4 h after challenge with anti-CD3 in vivo, whereas no statistically significant diet-related effect was apparent on the serum concentration of anti-IL-4 complex in the same animals (Fig. 3). The results shown in Fig. 3 were calculated by subtracting negative (isotype) control values averaging 27 and 17 pg/ml for IL-4 and IFN-γ, respectively.

It was 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 days), hence magnitude, was imposed.

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) prepubescent

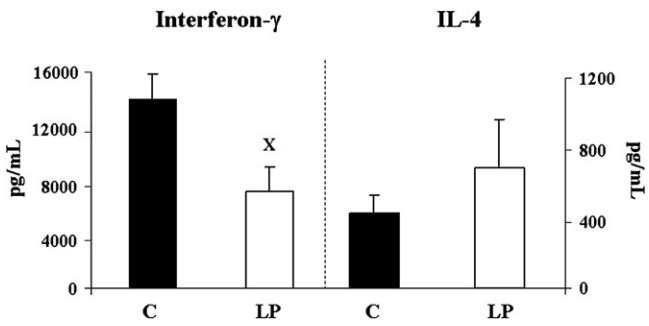

Fig. 3. Concentrations of IFN-γ and IL-4 complexed with their respective biotin-conjugated anti-cytokine antibody (R4-6A2 and BVD6-24G2) in the serum of C57BL/6J mice 4 h after intraperitoneal injection of the biotin-tagged probes together with 10 μg/mouse of anti-CD3 (145-2C11). Malnourished mice were given free access to a purified low-protein diet (group LP) during a 26-day 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 an S.E.M. value 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 ($P \leq .05$) according to two-tailed Student's *t* test.

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 4 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 prepubescent 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 time frames within which responses were assessed and on the basis of the stimulant that proved sufficient to elicit a response. First, to achieve effector status, naive T cells require more than the short response periods studied in the present investigation [8,9]. Second, naive 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 nonphysiological 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 therefore particularly important 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 prepubescent protein and/or energy deficit on the Types 1 and 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 prepubescent malnutrition, but, being evident in the low-protein model only in its extended (26 days) 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-γ 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 & Woodward, unpublished data). Furthermore, 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-γ 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-γ 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 prepubescent 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 prepubescent 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 prepubescent protein and energy deficit in their advanced stages; however, this index reliably reflects 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 reconfigured 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 but 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.

Acknowledgments

This work was supported by an operating grant (7333-03) awarded to B.D.W. through the Discovery Grants program by the Natural Sciences and Engineering Research Council of Canada. We thank Ms. Rinu Pazhekattu for providing technical assistance in connection with the carcass analyses reported herein.

References

[1] Collins S. Treating severe acute malnutrition seriously. Arch Dis Child 2007;92:453–61.
[2] Woodward B. The effect of protein–energy malnutrition on immune competence. In: Suskind RM, Tontisirin K, editors. Nutrition, immunity, and infection in infants and children. Philadelphia (Pa): Vevey/Lippincott Williams & Wilkins; 2001. pp. 89–116.
[3] Brundtland GH. Nutrition and infection: malnutrition and mortality in public health. Nutr Rev 2000;58:S1–4.
[4] Lucey DR, Clerici M, Shearer GM. Type 1 and type 2 cytokine dysregulation in human infectious, neoplastic, and inflammatory diseases. Clin Microbiol Rev 1996;9:532–62.
[5] Santana MA, Rosenstein Y. What it takes to become an effector T cell: the process, the cells involved, and the mechanisms. J Cell Physiol 2003;195:392–401.
[6] Gracie JA, Bradley JA. Interleukin-12 induces interferon-γ-dependent switching of IgG alloantibody subclass. Eur J Immunol 1996;26:1217–21.
[7] Szabo SJ, Sullivan BM, Peng SL, Glimcher LH. Molecular mechanisms regulating TH1 immune responses. Annu Rev Immunol 2003;21:713–58.
[8] Kaech SM, Wherry EJ, Ahmed R. Effector and memory T-cell differentiation: implications for vaccine development. Nature Rev Immunol 2002;2:251–62.
[9] Rogers PR, Dubey C, Swain SL. Qualitative changes accompany memory T cell generation: faster, more effective responses at lower doses of antigen. J Immunol 2000;164:2338–46.
[10] Dubey C, Croft M, Swain SL. Naïve and effector CD4 T cells differ in their requirements for T cell receptor versus costimulatory signals. J Immunol 1996;157:3280–9.
[11] Woodward B. Depressed adaptive immune competence in acute protein–energy malnutrition: a regulated pathophysiology controlled by endocrine hormones and cytokines. In: Chandra RK, editor. Nutrition and immunology in the 21st century. Toronto, Gurgaon: Tsar Health; 2004. pp. 23–38.
[12] Neyestani TR, Woodward B. Blood concentrations of Th2-type immunoglobulins are selectively increased in weanling mice subjected to acute malnutrition. Exp Biol Med 2005;230:128–34.
[13] Mengheri E, Nobili F, Crocchioni G, Lewis JA. Protein starvation impairs the ability of activated lymphocytes to produce interferon-γ. J Interferon Res 1992;12:17–21.
[14] Hill ADK, Hassan N, Shou J, Calvano SE, Daly JM. Antimicrobial effects of granulocyte-macrophage colony-stimulating factor in protein–energy malnutrition. Arch Surg 1995;130:1273–8.
[15] Chan J, Tian Y, Tanaka KE, Tanaka KE, Tsang MS, Yu K, et al. Effects of protein–calorie malnutrition on tuberculosis in mice. Proc Nat Acad Sci U S A 1996;93:14857–61.
[16] Finkelman FD, Morris SC. Development of an assay to measure in vivo cytokine production in the mouse. Int Immunol 1999;11:1811–8.
[17] Finkelman FD, Morris S, Orekhova T, Orekhova T, Sehy D. The in vivo cytokine capture assay for measurement of cytokine production in the mouse. In: Coligan JE, Bierer BE, Margulies DH, Shevach EM, Strober W, editors. Current protocols in immunology: Unit 6.28. New York (NY): John Wiley & Sons, Inc.; 2003. pp. 1–10.
[18] Woodward BD, Woods JW, Crouch DA. Direct evidence that acquired cell-mediated immunity is less resistant than is primary thymus-dependent humoral immunity to the depressive influence of wasting protein–energy malnutrition in weanling mice. Am J Clin Nutr 1992;55:1180–5.
[19] Lee WH, Woodward BD. The CD4/CD8 ratio in the blood does not reflect the response of this index in secondary lymphoid organs of weanling mice in models of protein–energy malnutrition known to depress thymus-dependent immunity. J Nutr 1996;126:849–59.
[20] Woodward B, Hillyer L, Hunt K. T cells with a quiescent phenotype ($CD45RA^+$) are overabundant in the blood and involuted lymphoid tissues in wasting protein and energy deficiencies. Immunol 1999;96:246–53.
[21] Ha CL, Wong SSL, Gray MM, Watt J, Hillyer LM, Woodward B. Overabundance of $CD45RA^+$ (quiescent-phenotype) cells within the involuted $CD4^+$ T-cell population follows initiation of immune depression in energy-deficient weanling mice and reflects involution exclusive to the $CD45RA^-$ subset. J Nutr 2001;131:1812–8.
[22] ten Bruggencate SJM, Hillyer LM, Woodward B. The proportion of $CD45RA^{+-}$ $CD62L^+$ (quiescent-phenotype) T cells within the $CD8^+$ subset increases in advanced weight loss in the protein- or energy-deficient weanling mouse. J Nutr 2001;131:3266–9.
[23] Zhang X, Hillyer LM, Woodward BD. The capacity of noninflammatory (steady-state) dendritic cells to present antigen in the primary response is preserved in acutely protein- or energy-deficient weanling mice. J Nutr 2002;132:2748–56.
[24] Filteau SM, Woodward B. The effect of severe protein deficiency on serum zinc concentration of mice fed a requirement level or a very high level of dietary zinc. J Nutr 1982;112:1974–7.
[25] Woods JW, Woodward BD. Enhancement of primary systemic acquired immunity by exogenous triiodothyronine in wasted, protein–energy malnourished weanling mice. J Nutr 1991;121:1425–32.
[26] Mittal A, Woodward B. Thymic epithelial cells of severely undernourished mice: accumulation of cholesteryl esters and absence of cytoplasmic vacuoles. Proc Soc Exp Biol Med 1985;178:385–91.
[27] Hillyer LM, Woodward B. Interleukin-10 concentration determined by sandwich enzyme-linked immunosorbent assay is unrepresentative of bioactivity in murine blood. Am J Physiol 2003;285:R1514–9.
[28] Shipp K, Woodward BD. A simple exsanguination method that minimizes acute pre-anesthesia stress in the mouse: evidence based on serum corticosterone concentrations. Contemp Topics Lab Anim Sci 1998;37:73–7.
[29] Konyer J, Hillyer LM, Woodward B. Splenic dendritic cell populations of the weanling mouse involute proportionately with total nucleated cell numbers throughout acute protein and energy deficiencies except in the most advanced stages of nitrogen-to-energy imbalance. Nutr Res 2003;23:921–31.
[30] Hillyer L, Dao B, Niemiec P, Lee S, Doidge M, Bemben I, et al. Elevated bioactivity of the tolerogenic cytokines, interleukin-10 and transforming growth factor-β, in the blood of acutely malnourished weanling mice. Exp Biol Med 2006;231:1439–47.
[31] Filteau SM, Woodward B. Influence of severe protein deficiency and of severe food intake restriction on serum levels of thyroid hormones in the weanling mouse. Nutr Res 1987;7:101–7.
[32] Adkins B, Leclerc C, Marshall-Clarke S. Neonatal adaptive immunity comes of age. Nature Rev Immunol 2004;4:553–64.
[33] Rosenblum MG, Unger BW, Gutterman JU, Hersh EM, David GS, Frincke JM. Modification of human leukocyte interferon pharmacology with a monoclonal antibody. Cancer Res 1985;45:2421–4.
[34] Jones AT, Ziltener HJ. Enhancement of the biologic effects of interleukin-3 in vivo by anti-interleukin-3 antibodies. Blood 1993;82:1133–41.
[35] Sato J, Hamaguchi N, Doken K, Gotoh K, Ootsu K, Iwasa S, et al. Enhancement of anti-tumor activity of recombinant interleukin-2 (rIL-2) by immunocomplexing with a monoclonal antibody against rIL-2. Biotherapy 1993;6:225–31.
[36] Courtney LP, Phelps JL, Karavodin LM. An anti-IL-2 antibody increases serum half-life and improves anti-tumor efficacy of human recombinant interleukin-2. Immunopharmacology 1994;28:223–32.
[37] Finkelman FD, Madden KB, Morris SC, Holmes JM, Boiani N, Katona IM, et al. Anti-cytokine antibodies as carrier proteins. Prolongation of in vivo effects of exogenous cytokines by injection of cytokine–anti-cytokine antibody complexes. J Immunol 1993;151:1235–44.
[38] Boyman O, Kovar M, Rubinstein MP, Surh CD, Sprent J. Selective stimulation of T cell subsets with antibody–cytokine immune complexes. Science 2006;311:1924–7.
[39] Kamimura D, Sawa Y, Sato M, Agung E, Hirano T, Murakami M. IL-2 in vivo activities and antitumor efficacy enhanced by an anti-IL-2 mAb. J Immunol 2006;177:306–14.
[40] Fernandez-Botran R, Vitetta ES. Evidence that natural murine soluble interleukin 4 receptors may act as transport proteins. J Exp Med 1991;174:673–81.
[41] Bendtzen K, Svenson M, Jonsson V, Hippe E. Autoantibodies to cytokines — friends or foes? Immunol Today 1990;11:167–9.
[42] Andrade MC, Vaz NM, Faria AMC. Ethanol-induced colitis prevents oral tolerance induction in mice. Braz J Med Biol Res 2003;36:1227–32.
[43] Westermann J, Pabst R. Distribution of lymphocytes and natural killer cells in the human body. Clin Invest 1992;70:539–44.
[44] Gonzalez-Martinez H, Rodriguez L, Najera O, Cruz D, Miliar A, Dominguez A, et al. Expression of cytokine mRNA in lymphocytes of malnourished children. J Clin Immunol 2008;28:593–9.
[45] Rodriguez L, Gonzalez C, Flores L, Jimenez-Zamudio L, Graniel J, Ortiz R. Assessment by flow cytometry of cytokine production in malnourished children. Clin Diagnost Lab Immunol 2005;12:502–7.

[46] Good RA, Lorenz E. Nutrition and cellular immunity. Int J Immunopharmac 1992; 14:361–6.
[47] Shi HN, Scott ME, Stevenson MM, Koski KG. Zinc deficiency impairs T cell function in mice with primary infection of *Heligmosomoides polygyrus* (Nematoda). Parasite Immunol 1994;16:339–50.
[48] Zhang S, Petro TM. The effect of moderate protein malnutrition on murine T cell cytokine production. Nutr Res 1997;17:51–64.
[49] Ing R, Su Z, Scott ME, Koski KG. Suppressed T helper 2 immunity and prolonged survival of a nematode parasite in protein-malnourished mice. Proc Nat Acad Sci U S A 2000;97:7078–83.