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Acutely malnourished weanling mice administered Flt3 ligand can support a cell-mediated inflammatory response

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ABSTRACT

The main objective of this investigation was to determine whether, despite acute (wasting) deficits of dietary nitrogen and energy, weanling mice could respond to the dendritic cell hematopoietin, Fms-like tyrosine kinase 3 ligand (Flt3L), in terms of an index of cell-mediated inflammatory competence. Male and female C57BL/6J weanlings were used, initially 19 days of age, and malnutrition was produced using a nitrogen-deficient diet. In preliminary work ten daily subcutaneous 1.0 μg doses of murine Flt3L, comparable to a protocol effective in humans, expanded the splenic conventional dendritic cell compartment ($CD11c^{+}F4/80^{-/low}$) of healthy weanlings without affecting the numbers of lymphocytes, macrophages, or recoverable mononuclear cells. Two subsequent experiments showed that, despite advancing malnutrition, exogenous Flt3L was able both to exert its classic influence on splenic conventional dendritic cell numbers and to invigorate the attenuated primary splenic cell-mediated inflammatory response to sheep erythrocytes. A final experiment showed that the cytokine intervention did not affect dendritic cell maturity according to several phenotypic indices. The findings provide new support for the proposition that dendritic cell numbers are the first limiting factor in the weak cell-mediated immune competence of acute pre-pubescent malnutrition. More substantially, intervention with Flt3L sustained an inflammatory systemic immune character despite progressive weanling malnutrition and weight loss. This outcome provides new support of fundamental character for the Tolerance Model which posits that the cell-mediated inflammatory incompetence of acute pre-pubescent protein and energy deficits is a regulated adaptive attempt, the antithesis of the classic paradigm of unregulated immunological attrition.

1. Introduction

According to a recent estimate [1] nearly 7,000,000 children lose their lives annually before the age of five years. Of this number 800,000 expire as a consequence of acute deficits of dietary protein and energy (i.e., wasting and/or edematous malnutrition) acting in synergy with infectious diseases [1], a toll of mortality undoubtedly exceeded numerically by an additional burden of infection-related morbidity. Immune depression is considered an important link between acute forms of pre-pubescent malnutrition and susceptibility to infection [2–5], and cell-mediated inflammatory incompetence occupies center stage in this regard as it has for decades [4,6]. Intervention to revive inflammatory capacities is one of three preferred, complementary approaches proposed almost twenty years ago with a view to reducing the burden of infection in pediatric protein and energy deficits [7]; yet, to date, little research effort has been directed toward this type of management strategy [5]. In this connection, however, rodent models of acute, pre-pubescent malnutrition have provided proof-of-concept that cell-mediated immune competence can be manipulated independently of ongoing weight loss in acute pre-pubescent malnutrition [2,6,8,9]. Hence, a grasp of malnutrition-associated immunological change should facilitate improved management of infection even in advanced stages of acute pediatric protein and energy deficit.

Understandably, the T cell has long governed the discussion of malnutrition-associated depression in cell-mediated immune competence [4,10]. However, appreciation of the cellular basis for the phenomenon remains primitive [3–5] and, in more recent years, the dendritic cell has emerged as a potentially decisive player [4,10]. Dendritic cells comprise a small population of immunological sentinels that mature within secondary lymphoid organs to become uniquely potent in presenting antigen to naïve T cells [11,12]. In fact, mature dendritic cell numbers are reported to limit primary T-dependent responses in the immunologically competent adult mouse [11,13]. This information base stimulated an investigation in which adoptive transfer of

Abbreviations: BSA, bovine serum albumin; Cy5, cyanine 5; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; Flt3L, Fms-like tyrosine kinase 3 ligand; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; PE, phycoerythrin; SRBC, sheep red blood cells

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syngeneic dendritic cells proved sufficient to restore primary cell-mediated inflammatory competence in a weanling mouse model of acute nitrogen and energy deficit [8]. Hence, at least in this model of pre-pubescent malnutrition, the spotlight is on the dendritic cell as a primary limiting factor to inflammatory immune competence. Moreover, important evidence from a study of acutely malnourished children similarly implicates the dendritic cell [4]. A clear basis exists, therefore, to pursue the dendritic cell in the effort to identify the cellular underpinning of the cell-mediated inflammatory incompetence that characterizes acute pre-pubescent malnutrition.

Fms-like tyrosine kinase 3 ligand (Flt3L) is essential and sufficient for homeostatic maintenance of dendritic cell compartments, a role fulfilled both by promoting development of hematopoietic progenitors and by stimulating proliferation of dendritic cells in the periphery [12,14,15]. Further, exogenous Flt3L expands dendritic cell populations of healthy rodents and humans [16–23], apparently exerting its influence in significant measure via conventional steady-state dendritic cell subsets. Importantly, the exogenous cytokine is effective even during early ontogeny, e.g. when administered to the mouse during the first postnatal week [22,23], a period that corresponds immunologically to the second trimester of human fetal development during which the bone marrow becomes the primary hematpoietic organ [24]. Although Flt3L also can promote expansion of macrophage and lymphocyte populations, the cytokine is particularly potent vis-à-vis the dendritic cell [20].

Classically, malnutrition-associated immune depression is considered to reflect an unregulated attrition of inflammatory capacities [2,6,9]. However, a growing body of research findings is difficult to reconcile with a model of immunological exhaustion [2]. This body of work includes reports that acutely malnourished rodents retain responsiveness to cytokines [9,25–28] and up-regulate constitutive production of key anti-inflammatory mediators [2,29]. These (and other) findings prompted the Tolerance Model proposition according to which acute forms of pre-pubescent malnutrition elicit a purposeful, i.e. regulated, restructuring toward a non-inflammatory form of immune competence [2,9,29,30]. Thus the Tolerance Model is the antithesis of the antecedent attrition/exhaustion paradigm. Ultimately, the Model suggests an attempt to minimize the inflammatory consequences of large-scale catabolic exposure to self-antigens, and its title highlights the broad possibility of this adaptive benefit.

This investigation was designed to complement a report that adoptive transfer of dendritic cells to acutely malnourished weanling mice restored a cell-mediated inflammatory response to sheep red blood cells (SRBC) that arises in the spleen [8]. The intervention throughout the present series of experiments was administration of murine Flt3L; therefore, a minor objective was to test a prediction of the Tolerance Model that exogenous Flt3L could expand the splenic compartment of conventional dendritic cells in the face of advancing protein and energy deficits. However, the Tolerance Model predicts more far-reaching possibilities up to, and including, plasticity of systemic inflammatory immune character despite unabated malnutrition and weight loss. To date, such a substantial prediction remains untested; hence, the main objective of this investigation centered on manipulation of a cell-mediated immune response in vivo, viz. the spleen-based delayed hypersensitivity response to SRBC. A final, corollary objective was to generate new evidence as to the centrality of the dendritic cell to the cell-mediated inflammatory depression of acute pre-pubescent malnutrition. It was anticipated that this investigation would provide a foundation for studies centered on the clinical goal of restoring resistance to infectious diseases in the face of wasting pediatric protein and energy deficits.

2. Materials and methods

2.1. Ethics statement

This investigation was performed under Animal Utilization Protocol 11R030 which was approved by the Animal Care Committee of the University of Guelph under the governance of the Canadian Council on Animal Care. Animal numbers were minimized to the full extent permitted by statistical rigour.

2.2. Animals, facilities, diets and feeding protocols

Male and female C57BL/6J mice were used from an in-house breeding colony, and husbandry practices are reported elsewhere [8]. The mice were weaned at 18 days of age and acclimated for one day with free access to a complete purified diet [31]. Subsequently the mice were assigned to consume, *ad libitum*, either the complete diet or an isocaloric low-protein formulation. The low-protein diet was formulated by replacement of much of the nitrogen source (spray-dried egg white) with corn starch and is described in full elsewhere [32]. Experimental periods were either 10 days or 14 days and coprophagy was permitted.

2.3. Preliminary experiment: a protocol for administering Flt3L to weanling mice

Protocols ranging from ten to fourteen daily subcutaneous doses of 10 μg Flt3L per kg of body weight are reported to increase dendritic cell numbers in the blood of adult human subjects [17,18]. Moreover, increasing the dose of cytokine ten-fold elicited no additional response [17]. Using this information as a reference point, an inter-species dose-translation calculation based on body surface area [33] yields an estimate of 1.2 μg Flt3L per day as equivalent for a mouse weighing 10 g. In previous studies of the adolescent and young adult mouse, however, the cytokine was administered subcutaneously for eight to ten days in single daily doses of 10 μg, and these protocols expanded populations of lymphocytes and mononuclear phagocytes in addition to the dendritic cell compartment [16,19,20]. Consequently, a preliminary experiment was performed to determine whether ten daily subcutaneous doses of 1.0 μg Flt3L (comparable physiologically to a protocol previously shown effective for humans [18]) would expand the splenic dendritic cell compartment of healthy weanling mice, preferably without influencing the numbers of mononuclear cells within other major subsets.

Eight weanling C57BL/6J mice, four males and four females, were given free access to the complete purified diet for 10 days, i.e. from 19 to 29 days of age. One group of two males and two females received ten daily interscapular subcutaneous injections of 1.0 μg murine Flt3L (the first at 19 days of age and the last at 28 days of age), whereas the remaining four mice were given daily sham injections. Spleen cell samples were taken 24 h after the final injection, and the primary outcome measure was splenic conventional dendritic cell numbers.

2.4. Three experiments with malnourished weanlings given exogenous Flt3L: Testing predictions of the Tolerance Model

(1) Four males and four females were fed the low-protein diet for ten days (19–29 days of age). Two males and two females received daily injections of Flt3L (the first at 19 days of age and the last at 28 days of age), whereas the remaining four mice served as shams. Blood and spleen cell samples were taken 24 h after the final injection, and the primary outcome measure was splenic conventional dendritic cell numbers.
(2) Three groups of ten mice were used (five males and five females per group). Two groups consumed the low-protein diet for 14 days (19–33 days of age), whereas the third group was fed the complete diet for the same period. One of the groups fed the low-protein diet

was given daily injections of Flt3L (the first at 19 days of age and the last at 28 days of age) and the other two groups were given sham injections. Within each group of ten mice, four males and four females (n = 8) were immunized with SRBC at 28 days of age, and one male and one female (n = 2) served as unsensitized controls. Blood and spleen cells were taken from the eight sensitized mice of each malnourished group when the anti-SRBC immune response, the primary outcome measure of the experiment, was assessed at 33 days of age. Tissue samples were not taken from the animals fed the complete diet because this group was intended only as a positive control for the immune response to SRBC.

(3) Three groups of six mice (four males and two females per group) were used. Two groups consumed the low-protein diet for ten days (19–29 days of age), whereas the third group was given the complete diet for the same period. One of the groups fed the low-protein diet was given daily injections of Flt3L (the first at 19 days of age and the last at 28 days of age) and the other two groups received sham injections. Blood and spleen cell samples were taken 24 h after the final injection, and the main outcome measures centered on dendritic cell maturity. So as to recover sufficient numbers of spleen cells, each of the six samples within the low-protein sham group comprised two mice pooled together. Likewise, two of the four samples of male animals within the group given Flt3L were made by pooling two mice. All pooling was done within sexes and each pooled sample was assigned a single degree of freedom.

2.5. Production of murine Flt3L and culture fluids for sham injections

Biohazards Project Number H-305-08-12 (Office of Research, University of Guelph) governed production of the cytokine. A plasmid designated pUMVC3-mFLex (Aldevron cat # 4031) was expanded in a culture of DH5α E. coli and the integrity of the plasmid was verified by means of restriction enzyme digestion. HEK293 cells (ATCC # CRL-1573) were transfected with the plasmid using 1,2-dioleoyl 1,2,3-trimethylammonium propane (Avanti Polar Lipids, cat # 890890C), and the transfected cells were cultured for 72 h without serum in Eagle's minimal essential medium supplemented with Earle's balanced salt solution. Non-transfected HEK293 cells were grown under the same conditions to provide negative control culture fluids for use in sham injections. The culture media were concentrated using Millipore Centricon Plus-70 centrifugation filters (MW cut-off 5 kDa, cat # UFC700508) after which the concentrates were subjected to SDS polyacrylamide gel electrophoresis followed by Western blotting, and their Flt3L content was quantified by ELISA. Western blots and the ELISA were validated against a murine Flt3L standard (R&D Systems, cat # 427-FL).

2.6. SDS polyacrylamide gel electrophoresis and western blotting of murine Flt3L

Concentrated culture fluids from transfected and non-transfected HEK293 cells were run through 15% acrylamide slab gels, each with a 5% stacking gel, using a Thermo EC 1000-90 electrophoresis unit. Each gel slab included a lane of recombinant murine Flt3L (R&D Systems, cat # 427-FL) and a lane of pre-stained proteins serving as a molecular weight ladder (Invitrogen, cat # 10748-010). The separated proteins were transferred to a PVDF membrane, blocked, and incubated with a polyclonal anti-mouse Flt3L (goat IgG, R&D Systems cat # AF427) followed by a peroxidase-conjugated donkey antibody against the heavy and light chains of goat IgG (Jackson Immunoresearch, cat # 705035147). The blocking buffer used prior to, and throughout, the staining steps contained 5% skim milk powder. Detection reagents were purchased from Amersham (cat # RPN2106V1) and the image was developed using a Kodak X-OMAT 1000A processor. The procedure yielded a major 18 kDa band together with a minor band, presumably a post-translational modification of the main protein, at a slightly higher molecular weight.

2.7. Sandwich ELISA for murine Flt3L

The assay was performed using 96-well round-bottom plates (Nunc Immunoplate, VWR cat # CA62407-162). The plates were coated with a polyclonal IgG-class goat anti-mouse Flt3L (R&D Systems, cat # AF427) and blocked with 10% fetal bovine serum (FBS, Sigma, cat # F4135) in phosphate-buffered saline (PBS, 0.1 M, pH 7.4). A biotin-conjugated polyclonal goat anti-mouse Flt3L (R&D Systems, cat # BAF427) served as the detection antibody which was visualized using streptavidin-conjugated horseradish peroxidase (BD Biosciences, cat # 51-26437E) together with a commercial substrate preparation (BD Biosciences, cat # 555214). Plates were read using a Vmax kinetic plate reader (Molecular Devices) at an absorbance of 450–570 nm. A commercial recombinant murine Flt3L (R&D Systems, cat # 427-FL) was used to generate the standard curve for each assay. According to described procedures [32], the intra-assay coefficient of variation ranged from 1.8% to 3.5% and the detection limit ranged from 4.6 to 5.9 pg/mL.

2.8. Administration of Flt3L

The concentrated culture fluids from transfected HEK 293 cells were diluted in sterile, endotoxin-free physiological saline to deliver 1.0 μg of Flt3L in a volume of 100 μL, and subcutaneous injections were given aseptically in the interscapular region. Culture fluids from non-transfected cells were prepared in an identical manner for sham injections.

2.9. Blood collection

Mice were anesthetized with CO_2 and terminally bled from the orbital plexus as described [34,35]. The blood was allowed to clot at room temperature for 30 min and, after centrifugation for four minutes at 12,000*g*, recovered serum was stored at −80 °C.

2.10. Isolation of splenic mononuclear cells

Following exsanguination, the spleen of each mouse was removed aseptically, minced in 5 mL RPMI 1640 medium (Sigma, cat # R8758) containing type 1A collagenase (1 mg/mL, Sigma, cat # C-9891) and 10% FBS (Sigma, cat # F4135) and incubated for 45 min at 37 °C. The tissue was then forced through a 100 mesh wire screen to yield a single-cell suspension which was layered onto 5 mL of Lympholyte-M (CedarLane, cat # CL5035). Following centrifugation (500*g* for 20 min at room temperature), the upper layer of cells was washed in 5 mL of 0.1 M PBS (pH 7.4) containing 1% bovine serum albumin (BSA, Boehringer, cat # 10 735 078 001). Finally, the washed cells were re-suspended in 2 mL of 1% BSA/PBS for counting in a Neubauer chamber, and cellular viability was determined using eosin Y exclusion.

2.11. Flow cytometry

Suspensions of splenic mononuclear cells, 250×10^3 (experiments centered on dendritic cell numbers) and 500×10^3 (experiment centered on dendritic cell maturity), were stained on ice for 30 min in a volume of 100 μL of 1% BSA/PBS, washed with 2 mL 1% BSA/PBS and re-suspended in 0.5 mL 1% BSA/PBS for flow cytometry using a Becton Dickenson FACSCalibur E4272 flow cytometer. Each analysis comprised 10^4 viable mononuclear cells (experiments centered on dendritic cell numbers), or $5–10 \times 10^3$ viable cells exhibiting a $CD11c^+F4/80^{-/low}$ surface phenotype (investigation of cellular maturity).

For the purpose of the investigations centered on splenic dendritic cell numbers appropriate marker reagents, including saturating quantities, were identified as follows:

(a) B cells - 0.5 μg phycoerythrin (PE)-conjugated anti-mouse CD19 (Cedarlane, cat # CL8914PE, mouse IgG2a, clone: 6D5),
(b) $CD4^+$ T cells - 0.25 μg PE-conjugated anti-mouse CD4 (Cedarlane,

cat # CL012PE, rat IgG2b, clone: GK1.5),
(c) $CD8^+$ T cells - 1 μg PE-conjugated anti-mouse CD8 (Cedarlane, cat # CL169PE, rat IgG2b, clone: YTS 169.4),
(d) macrophages - 0.5 μg fluorescein isothiocyanate (FITC)-conjugated anti-mouse F4/80 (Cedarlane, cat # CL8940F, rat IgG2b, clone: C1:A3-1) and
(e) dendritic cells - 0.25 μg PE-conjugated anti-mouse CD11c (Cedarlane, cat # CL8923PE, Armenian hamster IgG, clone: N418) together with 0.5 μg FITC-conjugated anti-mouse F4/80.

Each analysis included an isotype control, namely PE-conjugated mouse IgG2a (Cedarlane, cat # CLCMG2A04, clone: MOPC-173) for CD19, PE-conjugated rat IgG2b (Cedarlane, cat # CLCR2B04, clone RTK4530) for CD4, FITC-conjugated rat IgG2b (Cedarlane, cat # CLCR2B01, clone: KLH/G2b-1-2) for CD8 and F4/80, and PE-conjugated Armenian hamster IgG (eBiosciences, cat # 12-4888, clone: eBio299Arm) for CD11c.

For the purpose of the experiment centered on dendritic cell maturity, each sample yielded three cellular suspensions stained to identify both CD11c and F4/80. Subsequently, each suspension was stained with one of the following three reagents in the indicated saturating quantity:

(a) 0.25 μg PE-cyanine 5 (PE-Cy5)-conjugated anti-mouse MHCII (eBiosciences cat # 15-5322, rat IgG2b, clone: NIMR-4), or
(b) 0.5 μg PE-Cy5-conjugated anti-mouse CD83 (eBiosciences cat # 13-0831, rat IgG1, clone: Michel7), or
(c) 0.1 μg PE-Cy5-conjugated anti-mouse CD86 (eBiosciences cat # 15-0862, rat IgG2a, clone: GL1).

Each analysis was accompanied by the requisite PE-Cy5-conjugated isotype control, namely rat IgG2b (eBiosciences, cat #15-4031), rat IgG1 (eBiosciences, cat #15-4301), or rat IgG2a (eBiosciences, cat #15-4321).

2.12. Interpretation of surface marker analyses

Conventional dendritic cells and macrophages present a developmental continuum beginning with a common progenitor, and a definitive phenotypic distinction remains elusive [36]. CD11c is a classic pan-dendritic cell marker [36–38] but is also expressed at intermediate levels by some macrophages [36]. Conversely, to date F4/80 has served as the most definitive single identifier for murine macrophages [39] but some conventional dendritic cells also express this marker at a low level [36]. Therefore, in this investigation, mononuclear cells expressing a $CD11c^+F4/80^{-/low}$ surface phenotype were identified as conventional dendritic cells. Mononuclear cells expressing an $F4/80^+$ surface phenotype, defined herein to include $F4/80^{high}$ expression, were classified as macrophages regardless of their CD11c phenotype.

An $MHCII^{high}$ phenotype identifies mature conventional dendritic cells [40,41], i.e. cells having differentiated to emphasize antigen presentation rather than antigen uptake. Expression of CD86, another classic marker of maturity among conventional splenic dendritic cells of the mouse [40,41], also was used independently to identify mature cells, as was expression of CD83 which has emerged more recently as a maturity marker for conventional dendritic cells of the spleen and other lymphoid organs of the mouse [42].

Lymphocyte subsets were identified by means of CD19, CD4, or CD8. $CD19^+$ mononuclear cells were considered to be B cells because CD19 is expressed throughout the development of this cellular lineage beginning with the pro-B cell stage [43]. T cells were identified as either $CD4^+$ or $CD8^+$.

2.13. Interpretation of forward angle light scatter analyses

The flow cytometric index of forward angle light scatter is a useful first indicator of cellular viability [44] and was used to confine the analyses to viable cells. In addition, because this index relates to both cellular volume and cellular surface area [44], characteristics which increase as dendritic cells mature [45], forward scatter also was used as an independent measure of the global maturity of dendritic cell populations and subpopulations.

2.14. Delayed hypersensitivity response to sheep red blood cells (SRBC)

An aseptic intraperitoneal injection of 8×10^5 SRBC in 100 μL sterile, endotoxin-free physiological saline was given on the ninth day of the experimental period (i.e., at 28 days of age). On day 13, a challenge dose of SRBC (1.2×10^8) in endotoxin-free saline was given aseptically into the right hind footpad, and an equal volume of saline was injected into the contralateral hind footpad. The response (primarily accumulation of inflammatory fluid) was assessed 24 h later as the difference in maximum footpad thickness (challenged minus unchallenged) expressed as a percentage of the thickness of the unchallenged footpad. Unsensitized animals were included in assessing the response. These mice received an intraperitoneal injection of 100 μL of saline on day nine of the experimental period and their footpad response after SRBC challenge on day 13 was subtracted from the response of the sensitized mice to produce an estimate of lymphocyte-mediated inflammation.

2.15. Carcass analysis

Carcasses were stored at −20 °C and their dry matter and lipid contents were determined as described [8].

2.16. Statistics

The upper limit of probability for statistical significance was pre-set at $P = 0.05$ (two-tailed) throughout this investigation. Using the SAS system for Windows, version 8.2, data sets were first subjected either to a two-tailed Student's *t*-test or to a one-way ANOVA and the latter was followed, probability permitting ($P \leq 0.05$), by Tukey's Studentized Range test. Data sets that failed any of the four SAS tests for normality were subjected to non-parametric analysis, viz. the two-tailed Wilcoxon rank sum exact test for two-group comparisons and the Kruskal-Wallis test for multiple-group comparisons. If the Kruskal-Wallis test indicated differences among groups ($P \leq 0.05$), Dunn's Multiple Comparison test was applied.

For the purpose of the primary immunological outcome measures of each experiment, the sample sizes used permitted a statistical power of at least 0.9. Power-based sample size calculations [46] included assumptions that the smallest meaningful intervention-related difference in mean values was a factor of two (splenic dendritic cell numbers and the anti-SRBC response), 10% (% $MHCII^{high}$), 20% (% $CD86^+$ and % $CD83^+$), or 50 units (forward angle light scatter). The variance estimates required for these calculations were taken from our previous work including published reports [8,47,48].

3. Results

3.1. Exogenous Flt3L functioned as a dendritic cell hematopoietin when administered to weanling mice according to a protocol effective in human subjects

The serum concentration of Flt3L, assessed 24 h after the last injection of cytokine or sham supernatant, did not differ between the two groups of animals, an outcome consistent with the short half-life of Flt3L reported in the blood of the mouse [49]. Other key outcomes of

Table 1
Preliminary experiment in which murine Flt3 ligand was administered to weanling mice according to a dosage, schedule and route effective for humans.

Index	−Flt3 Ligand	+Flt3 Ligand	P Value[1]
Initial body weight (g)	8.1 ± 1.1	8.5 ± 0.7	0.53
Final body weight (g)	16.1 ± 2.6	15.8 ± 1.9	0.85
Food intake (g/g body weight/d)	0.34 ± 0.09	0.30 ± 0.05	0.46
Serum Flt3 ligand (pg/mL)	297 ± 28	240 ± 99	0.31
Splenic mononuclear cells[2]			
Total count ($\times 10^{-6}$)	53.1 ± 12.6	61.9 ± 13.8	0.39
$CD4^+$ ($\times 10^{-6}$)	4.9 (4.3–5.5)	3.7 (3.5–6.7)	0.34
$CD8^+$ ($\times 10^{-6}$)	2.7 (1.3–3.2)	3.5 (1.7–4.6)	0.20
$CD19^+$ ($\times 10^{-6}$)	13.8 (11.9–20.0)	14.3 (10.5–22.9)	0.83
$F4/80^+$ ($\times 10^{-6}$)	0.6 (0.3–1.0)	0.9 (0.7–1.4)	0.34
$CD11c^+F4/80^{-/low}$ ($\times 10^{-6}$)	1.1 ± 0.3	2.4 ± 0.5	0.003

Mean ± standard deviation, or median (range). Mice initially 19 days of age had free access to a complete diet and were administered by subcutaneous injection, once daily for 10 days (i.e., until 28 days of age), 1.0 μg recombinant murine Flt3 ligand ("+Flt3 Ligand"; HEK 293 cells) or supernatant from non-transfected HEK 293 cells ("−Flt3 Ligand"). The experiment was terminated 24 h after the last injection of Flt3 ligand or sham supernatant. n = 4 per group, two males and two females.

[1] P values are from either a two-tailed Student's *t*-test (comparison of means) or a two-tailed Wilcoxon rank sum exact test (nonparametric comparisons). $P \leq 0.05$ was considered statistically significant.

[2] Viable cells determined by eosin Y exclusion (total mononuclear cell count) or by forward angle light scatter (surface marker analyses).

the preliminary investigation are shown in Table 1. Growth indices were comparable to published values for C57BL/6J weanlings given free access to the purified diet used herein [47]. In addition, the number of viable splenic conventional dendritic cells ($CD11c^+F4/80^{-/low}$) recovered from the sham-injected mice was within expectations for the healthy mouse [50] and comprised a percentage of nucleated splenocytes within the expected range of 0.5–2% [15,23,40,51]. At the dosage and schedule of subcutaneous administration used herein, Flt3L elicited an increase in the numbers of splenic mononuclear cells exhibiting a $CD11c^+F4/80^{-/low}$ surface phenotype in healthy C57BL/6J weanlings without affecting the numbers of mononuclear cells determined to be $CD4^+$, $CD8^+$, $CD19^+$, or $F4/80^+$. Consequently this protocol, which is substantially similar to procedures shown to be effective in humans [17,18], was used throughout the present investigation.

3.2. Ad libitum consumption of the low-protein diet produced a wasting pathology

Key performance indices are shown in Table 2 with respect to the three experiments involving acutely malnourished animals. The complete diet supported food intakes, weight gains and whole-body compositions comparable to performances reported elsewhere [8,29,30,47,48,52–55]. By contrast, the imbalanced diet reduced nitrogen and caloric intake on a body weight basis, and the associated weight decrement has been shown [47,52–54] to reflect losses of both lean and fat tissue. In short, the low-protein diet elicited a wasting disease comparable to the pathology reported [8,48,54,55] in studies demonstrating depressed T-dependent inflammatory competence in the same experimental system. Exogenous Flt3L did not influence performance indices.

3.3. Acute weanling malnutrition did not affect the blood serum concentration of Flt3L

The blood serum Flt3L concentrations of sham-injected weanlings, both well-nourished (Tables 1 and 2) and malnourished (Table 2), were within the range of 100–500 pg/mL reported elsewhere for the mouse [56,57]. Importantly, a direct comparison between well-nourished and malnourished groups (Experiment 3) revealed no influence of acute protein and energy deficits at the advanced stage of weight loss examined (Table 2). Notably, also, the concentration of Flt3L in the blood serum of malnourished mice was elevated as much as an order of magnitude 24 h after the last injection of cytokine (Table 2), whereas no effect on the blood Flt3L concentration was apparent 24 h after the last injection of cytokine given to well-nourished animals (Table 1).

3.4. Exogenous Flt3L expanded the $CD11c^+F4/80^{-/low}$ compartment in the spleen of acutely malnourished weanling mice

The exogenous cytokine exerted no influence on the number of mononuclear cells recovered from the spleen of acutely malnourished weanling animals and no influence on the numbers of recoverable $CD4^+$, $CD8^+$, $CD19^+$ or $F4/80^+$ splenic mononuclear cells (Table 3). By contrast, the number of cells exhibiting a $CD11c^+F4/80^{-/low}$ surface phenotype was several-fold higher in the group of animals given exogenous Flt3L than in the negative control cohort (Table 3). Importantly, a selective influence of the exogenous cytokine on the dendritic cell compartment remained apparent when the cell counts within the major subsets were expressed as percentages of the viable splenic mononuclear cell population (Table 3). This selectivity of influence, achieved in both healthy and acutely malnourished weanlings (Tables 1 and 3, respectively), contrasts with previous rodent-based studies [16,19,20] in which the daily dose of cytokine was an order of magnitude higher than the dose administered in the present investigation.

3.5. Exogenous Flt3L increased the adaptive cell-mediated response to SRBC in weanling mice subjected to acute protein and energy deficit

Five days after the last of ten daily injections of Flt3L or sham supernatant the group given exogenous cytokine yielded a larger number of viable splenic mononuclear cells than the sham-injected controls (Table 4). This outcome reflected an increase in the numbers of several types of hematopoietic cells; thus, an impact of Flt3L on key spleen cell subsets was apparent following the sensitization and challenge periods of this experiment, i.e. five days after the last dose of exogenous cytokine. Of the subsets monitored, however, only that exhibiting a $CD11c^+F4/80^{-/low}$ surface phenotype constituted a higher percentage of the total mononuclear cell population following administration of Flt3L (Table 4).

The primary cell-mediated inflammatory responses to SRBC are shown in Fig. 1 which presents the increases in footpad thickness as percentage values so as to accommodate the inevitable malnutrition-related differences in footpad thickness. The responses were determined by subtracting estimates of the nonspecific response to challenge exhibited by unsensitised animals (complete diet: 5.9%, n = 2; low-protein diet: 4.0%, n = 2 from each group of malnourished mice, by inspection no effect of Flt3L). Comparison with the sham-injected positive control group (complete diet) revealed that the sham-injected malnourished group could mount only a weak response as shown previously [8,48] in this model of acute weanling malnutrition. By contrast, malnourished animals given Flt3L mounted a vigorous inflammatory footpad response which did not differ from that of the positive control group (complete diet). Likewise, comparison of the absolute values of footpad thickness increases (mean [SD], corrected for nonspecific response: 0.14 [0.07], 0.35 [0.09], 0.48 [0.22] mm, respectively, in the groups designated sham-injected low-protein diet, cytokine-injected low-protein diet and sham-injected complete diet) revealed that the malnourished group given exogenous Flt3L not only supported a more vigorous footpad response than the sham-injected malnourished group but also, despite their small footpads, generated an immunologically-mediated increase in thickness that did not differ from the response of the group fed the complete diet ($P = 0.001$, Tukey's Studentized Range test). This outcome dovetails with the finding (Table 4) that, five days after sensitization, splenic cellular subsets required for the

Table 2
Performance indices and serum Flt3 ligand concentrations.

Index	Complete Diet	Low-protein Diet		P Value[1]
		−Flt3 Ligand	+Flt3 Ligand	
Experiment 1:Influence of Flt3 Ligand on Splenic Dendritic Cell Numbers in Malnourished Mice				
Initial body weight (g)	–	8.3 ± 0.6	8.7 ± 0.9	0.46
Final body weight (g)	–	6.6 ± 0.3	6.8 ± 0.7	0.59
Food intake (g/g body wt/d)	–	0.20 ± 0.04	0.17 ± 0.03	0.29
Carcass dry matter (%)	–	28.6 ± 0.9	26.5 ± 3.0	0.24
Carcass lipid (%)	–	3.3 ± 0.8	3.1 ± 1.0	0.68
Serum Flt3 ligand (pg/mL)	–	260 ± 40	1350 ± 672	0.02[2]
Experiment 2: Influence of Flt3 Ligand on Anti-SRBC Delayed Hypersensitivity in Malnourished Mice				
Initial body weight (g)	8.1 ± 0.5	8.4 ± 0.6	8.2 ± 0.8	0.63
Final body weight (g)	18.6^{a} ± 1.0	6.5^{b} ± 0.4	6.3^{b} ± 0.5	0.0001
Food intake (g/g body wt/d)	0.34^{a} ± 0.01	0.21^{b} ± 0.02	0.21^{b} ± 0.02	0.0001
Carcass dry matter (%)	32.0 ± 4.0	28.9 ± 2.7	29.7 ± 4.4	0.13
Carcass lipid (%)	8.8^{a} ± 1.3	3.6^{b} ± 0.6	3.9^{b} ± 1.1	0.0001
Serum Flt3 ligand (pg/mL)[3]	–	211 ± 59	155 ± 53	0.07
Experiment 3: Influence of Flt3 Ligand on Splenic Dendritic Cell Maturity in Malnourished Mice				
Initial body weight (g)	8.0 ± 0.9	8.1 ± 0.6	8.4 ± 0.9	0.65
Final body weight (g)	14.9^{a} ± 1.4	6.7^{b} ± 0.5	7.2^{b} ± 0.7	0.0001
Food intake (g/g body wt/d)	0.33^{a} ± 0.04	0.21^{b} ± 0.02	0.20^{b} ± 0.01	0.0001
Serum Flt3 ligand (pg/mL)	120^{a} ± 55	120^{a} ± 48	1300^{b} ± 210	0.001

Mean ± standard deviation. Mice initially 19 days of age had free access to either a complete diet or a low-protein diet and were administered by subcutaneous injection, once daily for 10 days (i.e., until 28 days of age), 1.0 μg recombinant murine Flt3 ligand ("+Flt3 Ligand"; HEK 293 cells) or supernatant from non-transfected HEK 293 cells (groups designated "Complete Diet and "Low-protein Diet −Flt3 Ligand"). Experiments 1 and 3: 10-day feeding period, terminated 24 h after final injection of Flt3 ligand or sham supernatant; n = 4 and 6, respectively, per group. Experiment 2: 14-day feeding period, terminated five days after the last cytokine or sham injection, i.e. five days after sensitization with sheep red blood cells (SRBC) and 24 h after challenge with SRBC to elicit a delayed hypersensitivity response; n = 10 per group, five males and five females, each group including one male and one female not sensitized with SRBC.

[1] P values are from a two-tailed Student's *t*-test (two groups) or a one-way ANOVA (three groups). P ≤ 0.05 was considered significant. Within a row showing multiple comparisons, mean values not sharing a superscript letter differ statistically (P ≤ 0.05) according to Tukey's Studentized Range test.

[2] *t*-test for unequal variances.

[3] Animals sensitized with sheep erythrocytes, only, i.e. n = 8 per group, four males and four females.

Table 3
Experiment 1: Influence of exogenous murine Flt3 ligand on numbers of viable mononuclear cells recovered from major splenic subsets of acutely malnourished weanling mice.

Index	−Flt3 Ligand	+Flt3 Ligand	P value[1]
Numbers of viable mononuclear cells			
Total cell count ($\times 10^{-6}$)	3.6 ± 1.6	4.5 ± 2.6	0.60
$CD4^{+}$ ($\times 10^{-3}$)	517 ± 250	439 ± 326	0.72
$CD8^{+}$ ($\times 10^{-3}$)	195 ± 100	312 ± 270	0.45
$CD19^{+}$ ($\times 10^{-3}$)	1333 ± 624	970 ± 836	0.51
$F4/80^{+}$ ($\times 10^{-3}$)	88 ± 54	107 ± 74	0.69
$CD11c^{+}F4/80^{-/low}$ ($\times 10^{-3}$)	125 (20–147)	360 (153–753)	0.03
Percentage of Viable Mononuclear Cells			
$CD4^{+}$	14.0 ± 1.5	11.0 ± 8.0	0.52[2]
$CD8^{+}$	5.4 ± 0.8	7.5 ± 6.4	0.56[2]
$CD19^{+}$	36.5 ± 8.3	23.0 ± 20.1	0.30[2]
$F4/80^{+}$	2.5 ± 1.1	2.3 ± 1.3	0.80
$CD11c^{+}F4/80^{-/low}$	3.0 (1.4–3.3)	8.3 (4.8–11.5)	0.03

Mean ± standard deviation, or median (range). Mice initially 19 days of age had free access to a low-protein diet and were administered by subcutaneous injection, once daily for 10 days (i.e., until 28 days of age), 1.0 μg recombinant murine Flt3 ligand ("+Flt3 Ligand"; HEK 293 cells) or supernatant from non-transfected HEK 293 cells ("−Flt3 Ligand"). The experiment was terminated 24 h after the last injection of Flt3 ligand or sham supernatant. Cellular viability was determined by eosin Y exclusion (total mononuclear cell count) or by forward angle light scatter (surface marker analyses). n = 4 per group, two males and two females.

[1] P values are from either a two-tailed Student's *t*-test (comparison of means) or a two-tailed Wilcoxon rank sum exact test (non-parametric comparisons). P ≤ 0.05 was considered statistically significant.

[2] *t*-test for unequal variances.

Table 4
Experiment 2: Influence of exogenous Flt3 ligand on numbers of viable mononuclear cells recovered from major splenic subsets of acutely malnourished weanling mice sensitized to elicit a delayed hypersensitivity response to sheep erythrocytes.

Index	−Flt3 Ligand	+Flt3 Ligand	P value[1]
Numbers of viable mononuclear cells			
Total count ($\times 10^{-6}$)	1.4 (0.7–3.4)	2.4 (1.2–5.4)	0.05
$CD4^{+}$ ($\times 10^{-3}$)	243 (105–500)	335 (171–684)	0.04
$CD8^{+}$ ($\times 10^{-3}$)	61 (41–253)	118 (43–370)	0.05
$CD19^{+}$ ($\times 10^{-3}$)	623 (391–1530)	820 (165–1890)	0.40
$F4/80^{+}$ ($\times 10^{-3}$)	22 (9–59)	43 (27–155)	0.05
$CD11c^{+}F4/80^{-/low}$ ($\times 10^{-3}$)	72 ± 22	200 ± 66	0.01
Percentage of viable mononuclear cells			
$CD4^{+}$	15.7 ± 1.3	14.3 ± 2.0	0.13
$CD8^{+}$	5.1 ± 1.2	5.3 ± 1.4	0.76
$CD19^{+}$	37.4 ± 10.2	32.2 ± 8.6	0.19
$F4/80^{+}$	1.9 ± 0.9	2.1 ± 0.6	0.62
$CD11c^{+}F4/80^{-/low}$	5.6 (2.1–6.5)	8.4 (5.5–9.4)	0.01

Mean ± standard deviation, or median (range). Mice initially 19 days of age had free access to a low-protein diet and were administered by subcutaneous injection, once daily for 10 days (i.e., until 28 days of age), 1.0 μg recombinant murine Flt3 ligand ("+Flt3 Ligand"; HEK 293 cells) or supernatant from non-transfected HEK 293 cells ("−Flt3 Ligand"). The experiment required a 14-day feeding period and was terminated five days after the last cytokine or sham injection, i.e. five days after sensitization with sheep red blood cells and 24 h after challenge with sheep erythrocytes to elicit a delayed hypersensitivity response. Cellular viability was determined by eosin Y exclusion (total mononuclear cell count) or by forward angle light scatter (surface marker analyses). n = 8 per group, four males and four females, i.e. excludes the two mice of each group that were not sensitized with sheep red blood cells.

[1] P values are from either a two-tailed Student's *t*-test (comparison of means) or a two-tailed Wilcoxon rank sum exact test (non-parametric comparisons). P ≤ 0.05 was considered statistically significant.

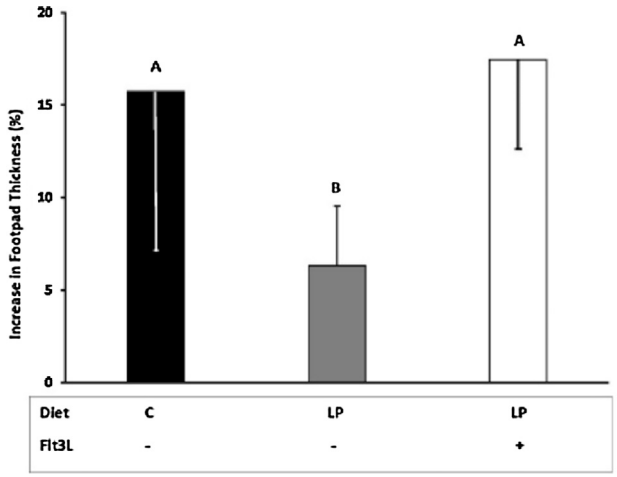

Fig. 1. Delayed hypersensitivity response to SRBC. Mice initially 19 days of age had free access for 14 days to a complete diet (C) or to a low-protein diet (LP). One malnourished group was administered, by subcutaneous injection, 1.0 μg of recombinant Flt3 ligand (HEK 293) once daily for 10 days (i.e., until 28 days of age), and the other two groups were given sham doses of supernatant from non-transfected HEK 293 cells. Each mouse was given an immunizing dose of SRBC at the time of the last injection of Flt3 ligand or sham supernatant and, four days later, each animal was challenged by injection of the sensitizing antigen into the right hind footpad. The response was assessed 24 h later, i.e. at 33 days of age, in terms of the thickness of the challenged footpad relative to the unchallenged contralateral footpad. n = 8 per group, four males and four females. Means are shown together with standard deviation bars, and are corrected for the nonspecific response of unsensitised animals. A one-way ANOVA (P = 0.003) permitted post hoc testing, and differing upper case letters identify differences (P ≤ 0.05) according to Tukey's Studentized Range test.

anti-SRBC response were expanded in animals given Flt3L relative to the same subsets in the sham-injected negative controls.

3.6. Neither acute malnutrition nor exogenous Flt3L influenced the phenotypic maturity of the conventional dendritic cell compartment in the spleen of weanling mice

The malnutrition protocol used herein elicited the characteristic reduction reported elsewhere [32] in numbers of splenic mononuclear cells, and this predictable outcome was unaffected by intervention with Flt3L (not shown). Notably, however, the reduction in numbers of mononuclear cells exhibiting a $CD11c^{+}F4/80^{-/low}$ surface phenotype was proportionate with the global involution of the splenic mononuclear cell compartment (Table 5). Exogenous Flt3L increased the numbers of mononuclear cells exhibiting a $CD11c^{+}F4/80^{-/low}$ surface phenotype in the spleen of the malnourished animals and also increased the percentage of mononuclear cells represented by this phenotype (Table 5), thus confirming previous findings of this investigation (Table 3). Adding emphasis to this outcome, the malnourished animals given Flt3L yielded numbers of spleen cells with a $CD11c^{+}F4/80^{-/low}$ phenotype that did not differ from the numbers recovered from sham-injected mice fed the complete diet (Table 5).

The maturity of splenic $CD11c^{+}F4/80^{-/low}$ mononuclear cell populations was assessed according to four phenotypic indices, viz. forward angle light scatter (relating to cellular volume and surface area [44] and, hence, reflecting dendritic morphology) and the percentage of cells exhibiting $MHCII^{high}$, $CD86^{+}$, or $CD83^{+}$ surface phenotype (Table 5). Neither the low-protein diet nor administration of Flt3L exerted a discernible influence on these indices.

Within the subpopulations of spleen cells identified as $MHCII^{high}$, $CD86^{+}$, or $CD83^{+}$, the expression density of the marker provides an index of cellular maturation [40–42]. In turn, fluorescence intensity can be used to quantify the expression density of a surface marker if appropriate attention is given to the factor of cellular surface area. In this investigation, forward angle light scatter (hence, by interpretation, cellular surface area [44]) was not affected by malnutrition or exogenous Flt3L within the $MHCII^{high}$, $CD86^{+}$, or $CD83^{+}$ subpopulations of splenic $CD11c^{+}F4/80^{-/low}$ mononuclear cells (Table 6). This outcome permitted comparisons of fluorescence intensities, and no diet- or cytokine-related differences emerged for any of the three cellular surface markers (Table 6).

Phenotypic indices of maturity, therefore, revealed no differences attributable to acute malnutrition or exogenous Flt3L either within the full population of spleen cells defined as $CD11c^{+}F4/80^{-/low}$ (Table 5) or within three subsets of this population identified by means of maturity-related surface markers (Table 6). It should be noted that the dendritic cells of our weanling animals exhibited an adult level of phenotypic maturity (Table 5) as reported elsewhere on the part of weanling mice [23].

Table 5
Experiment 3: Influence of acute malnutrition and exogenous murine Flt3 ligand on numbers and four phenotypic maturity indices of $CD11c^{+}F4/80^{-/low}$ spleen cells from weanling mice.

Index	Complete Diet	Low-protein Diet		P Value[1]
		−Flt3 Ligand	+Flt3 Ligand	
$CD11c^{+}F4/80^{-/low}$ ($\times 10^{-6}$)	0.68^{a} ± 0.17	0.12^{b} ± 0.02	0.65^{a} ± 0.22	0.0001
$CD11c^{+}F4/80^{-/low}$ (%)[2]	2.1 (1.4–3.0)^{a}	3.0 (2.0–3.9)^{a}	9.2 (5.7–11.4)^{b}	0.002
Forward scatter[3]	593 ± 20	557 ± 25	572 ± 30	0.08
$MHCII^{high}$ (%)[4]	90 ± 5	91 ± 3	89 ± 3	0.82
$CD86^{+}$ (%)[4]	62 ± 6	54 ± 7	58 ± 4	0.11
$CD83^{+}$ (%)[4]	17 ± 6	16 ± 5	11 ± 4	0.18

Mean ± standard deviation, or median (range). Mice initially 19 days of age had free access to either a complete diet or a low-protein diet and were administered by subcutaneous injection, once daily for 10 days (i.e., until 28 days of age), 1.0 μg recombinant murine Flt3 ligand ("+Flt3 Ligand"; HEK 293 cells) or supernatant from non-transfected HEK 293 cells (groups designated "Complete Diet" and "Low-protein Diet −Flt3 Ligand"). The experiment was terminated 24 h after the last injection of Flt3 ligand or sham supernatant. The outcomes shown are based on viable cells as discerned by forward angle light scatter. n = 6 per group, four males and two females. Each of the six samples within the low-protein sham group comprised two mice pooled together and assigned a single degree of freedom. Likewise two of the four samples of male animals within the group given Flt3 ligand were formed by pooling two mice.

[1] P values are from a one-way ANOVA (comparison of means) or a Kruskal-Wallis test (non-parametric comparisons). A statistical probability of P ≤ 0.05 was considered significant. Within a row, values not sharing a superscript letter differ according to Tukey's Studentized Range test (ANOVA) or Dunn's Multiple Comparison test (Kruskal-Wallis).

[2] Percentage of viable splenic mononuclear cells.

[3] Arbitrary units.

[4] Percentage of the cellular population exhibiting $CD11c^{+}F4/80^{-/low}$ surface phenotype.

Table 6
Experiment 3: Influence of acute malnutrition and exogenous murine Flt3 ligand on phenotypic indices of the maturity of MHCII^{high}, CD86^{+}, or CD83^{+} subsets of CD11c^{+}F4/80$^{-/low}$ mononuclear cells recovered from the spleen of weanling mice.

Subset	Complete Diet	Low-protein Diet		P value[1]
		−Flt3 Ligand	+Flt3 Ligand	
Forward angle light scatter[2]				
MHCII^{high}	593 ± 21	554 ± 26	566 ± 32	0.06
CD86^{+}	664 ± 32	646 ± 39	644 ± 50	0.64
CD83^{+}	687 ± 37	692 ± 21	672 ± 15	0.39
Fluorescence intensity[2]				
MHCII^{high}	931 (642–1057)	774 (519–894)	738 (425–886)	0.15
CD86^{+}	83 ± 19	76 ± 14	67 ± 15	0.25
CD83^{+}	93 (68–155)	98 (56–334)	94 (73–127)	0.99

Mean ± standard deviation, or median (range). Mice initially 19 days of age had free access to either a complete diet or a low-protein diet and were administered by subcutaneous injection, once daily for 10 days (i.e., until 28 days of age), 1.0 μg recombinant murine Flt3 ligand ("+Flt3 Ligand"; HEK 293 cells) or supernatant from non-transfected HEK 293 cells (groups designated "Complete Diet" and "Low-protein Diet −Flt3 Ligand"). The experiment was terminated 24 h after the last injection of Flt3 ligand or sham supernatant. n = 6 per group, four males and two females. Each of the six samples within the low-protein sham group comprised two mice pooled together and assigned a single degree of freedom. Likewise two of the four samples of male animals within the group given Flt3 ligand were formed by pooling two mice. The outcomes shown are based on viable cells as discerned by forward angle light scatter.

[1] P values are from either a one-way ANOVA (comparison of means) or the Kruskal-Wallis test (non-parametric comparisons). A statistical probability of $P \leq 0.05$ was considered significant.

[2] Arbitrary units.

4. Discussion

The form of weanling malnutrition produced herein was selected because it exhibits the classic cell-mediated inflammatory immune depression of acute pediatric malnutrition [8,48,55], and because it is relevant both immunologically and metabolically to incipient kwashiorkor [58]. Likewise, the cell-mediated anti-SRBC response was selected because it represents the type of inflammatory competence that is consistently depressed by acute weanling malnutrition [4,6], because its cellular requirements and kinetics resemble the classic tuberculin reaction [59] and because it arises within the spleen [60], the largest inflammatory lymphoid organ of mammalian animals. In addition, use of the anti-SRBC response provided continuity between this investigation and its antecedent [8] in which adoptive transfer of dendritic cells restored the same index of cell-mediated inflammatory competence in the same weanling model of acute malnutrition. Finally, murine Flt3L was used because, in the mouse, this cytokine particularly promotes type-1 polarized dendritic cells which most potently stimulate cell-mediated inflammatory responses [19]. Remarkably, a non-invasive intervention with this cytokine, alone, sustained cell-mediated inflammatory competence in a relevant model of acute pre-pubescent malnutrition (Fig. 1). It is of interest that Flt3L could fulfill its classic role as a dendritic cell growth factor in the face of acute weanling malnutrition when administered according to a dosage, schedule and route comparable physiologically to a protocol [18] in which the cytokine served as a human dendritic cell hematopoietin. However, the centerpiece of this investigation is its proof-of-concept that, as predicted by the Tolerance Model of malnutrition-associated inflammatory depression [2,9,29,30], systemic immunological plasticity remains despite advanced and ongoing wasting malnutrition even during the pediatric stage of life. As a corollary outcome, Flt3L is added to the growing list of cytokines for which the blood concentration is sustained in advanced stages of acute pre-pubescent malnutrition. The findings reported herein provide a foundation for studies aimed at mechanisms and pre-clinical questions such as pathogen resistance.

This investigation centers on the conventional dendritic cell and excludes the plasmacytoid subset which, although Flt3L-sensitive [61], exhibits a CD11c^{low} surface phenotype [51]. The findings reported here (Table 5) confirm a previous report [54] that acute weanling deficits of protein and energy reduce the numbers of dendritic cells within secondary lymphoid organs in proportion with the global involution of the mononuclear cell compartment. In view of the rapid turnover of dendritic cells in the spleen of the mouse [15,40], therefore, this cellular compartment exhibits a noteworthy resistance to malnutrition-induced involution. Further, to the extent that phenotypic indices can inform, malnutrition did not affect the steady state maturity of the splenic conventional dendritic cell compartment in this investigation (Tables 5 and 6). The latter outcome is consistent with a report that the antigen-presenting capacity of splenic and nodal dendritic cells, assessed both in vitro and in vivo, was unaffected by acute weanling malnutrition imposed either according to the protocol used herein or in a metabolically dissimilar form [54]. In apparent contrast, presumptive splenic dendritic cells of adult mice subjected to chronic caloric deficit exhibited a decline not only in numbers but also in antigen-presenting capacity and cytokine production in vitro [62]. However, an insightful analysis of blood dendritic cells from acutely malnourished children emphasized consistent numerical decline (absolute values and percentage of mononuclear cells) while revealing an influence on cellular maturity, according to surface markers and functional indices, that was confined to a minor subgroup of subjects exhibiting endotoxemia [4]. It is of interest that the surface marker indices used herein included expression density of MHCII, CD86 and CD83 which, in the latter report [4], mirrored the cytokine production profile and antigen-presenting capacity of corresponding dendritic cell populations and subsets [4]. All things considered, the findings reported here are consistent with the perspective [4] that the first influence of acute pre-pubescent malnutrition on the dendritic cell compartment is to reduce cellular numbers. In this connection, a low dendritic cell count emerged as a particularly potent independent predictor of mortality within a cohort of acutely malnourished children at risk of succumbing to infectious disease [4].

In the form of acute weanling malnutrition examined here, exogenous Flt3L proved able to fulfill its mandate as a dendritic cell hematopoietin with respect to the conventional dendritic cells resident within the spleen (Tables 3–5). Importantly, in the absence of the confounding factor of immunization, the exogenous cytokine elicited an increase in the proportion of conventional dendritic cells within the splenic mononuclear cell compartment without influencing the proportion represented by any other major subsets of mononuclear cells (Table 3). Moreover, the increase in cellular numbers was achieved in the absence of an influence on the global steady state phenotypic maturity of the dendritic cell population assessed both according to expression of markers including critical T cell ligands and according to an index reflecting dendritic morphology (Tables 5 and 6). In apparent contrast, exogenous Flt3L elicited a maturation response on the part of the phenotypically immature splenic dendritic cell compartment of the mouse during the first seven postnatal days [23]; however, the outcome reported in the present investigation is consistent with reports pertaining to the adolescent and young adult mouse [16,63] in which the exogenous cytokine increased dendritic cell numbers in the spleen and other lymphoid organs without affecting global steady state cellular maturity within the dendritic cell compartment. Indices of maturity in the latter reports [16,63] included several functional indicators both in vitro and in vivo in addition to surface phenotypic indices encompassing the markers used herein. Further, the same outcome is reported in a mouse model of transgenic overexpression of Flt3L [57]. Evidently the classic response of the weaned mouse to Flt3L as a dendritic cell growth factor was preserved, at least qualitatively, into the advanced stages of the form of acute weanling malnutrition produced in this

investigation. It is also of interest that, despite the rapid turnover of splenic conventional dendritic cells which exhibit a half-life of two or three days in the mouse [15,40], an expanded dendritic cell compartment was sustained through five days of advancing malnutrition and weight loss after the cytokine intervention was terminated (Table 4).

A scant but intriguing literature documents responsiveness to four cytokines, viz. interleukin-1 and granulocyte-, macrophage- and granulocyte-macrophage colony stimulating factors, by a handful of innate defence components in the face of acute protein and energy deficits [25–28]. This information base is expanded by the present investigation to include Flt3L together with responsiveness on the part of an index relating to adaptive immune competence. In addition, previous reports were limited to malnourished adults [25–28], whereas the weanling model used herein falls within an ontogenetic interval identified by Veru et al. [24] as a window of vulnerability in the development of an, as yet, incomplete adaptive immune assemblage. The particular importance of targeting an appropriate stage of ontogeny has been emphasized recently in relation to animal modeling of acute prepubescent malnutrition [5,9]. This investigation, therefore, confers new strength to the Tolerance Model by examining a previously untested cytokine, using an index of adaptive inflammatory competence and centering on a fragile pre-pubescent stage of immunological development.

In previous work [8], adoptive transfer of syngeneic dendritic cells was sufficient to restore the cell-mediated anti-SRBC response at the same stage of the same weanling malnutrition pathology as that studied here. The implication was that conventional dendritic cell numbers are the first limiting factor in the diminished primary cell-mediated inflammatory competence that characterizes acute pre-pubescent malnutrition. Similarly, considered collectively, the findings of the investigation reported here appear to connect the invigoration of the splenic anti-SRBC response by exogenous Flt3L (Fig. 1) with the cytokine-induced increase in splenic conventional dendritic cell numbers at the time selected for sensitization with SRBC (Tables 3 and 5). In a simple three-point interpretation, exogenous Flt3L first expanded the splenic dendritic cell compartment both in absolute numerical terms and in numbers relative to T cells (Table 3). This increased the capacity to present SRBC-derived antigens to naïve T cells, the rate-limiting step in primary cell-mediated adaptive responses at least in the healthy mouse [11,13]. Consequently (second), splenic T cell populations of the cytokine-treated animals, although unaffected numerically prior to sensitization (Table 3), expanded under the influence of enhanced presentation of SRBC-derived antigens (Table 4). Finally (third), the exogenous cytokine increased the anti-SRBC response with neither evidence of an influence on the global maturity of the splenic dendritic cell compartment at the moment of sensitization (Tables 5 and 6) nor reason to anticipate such an influence [16,57,63]. Thus, it is probable that the action of exogenous Flt3L to increase conventional dendritic cell numbers in the spleen was sufficient, in the present investigation, to invigorate an inflammatory anti-SRBC response that arises within this lymphoid organ [60]. The findings reported here, therefore, provide independent evidence for the need, highlighted elsewhere [4,8,10], to shift the focus of attention from the T cell system to the dendritic cell compartment in the effort to understand the cellular basis for depressed cell-mediated immune competence in acute pre-pubescent deficits of nitrogen and energy. Emphasis is added to this point by noting that dendritic cells are the unique antigen-presenting element for naïve T cells [11,12] which, in turn, are overabundant relative to the effector/memory type in diverse forms of acute pre-pubescent malnutrition [6,32].

The core proposition of the Tolerance Model is that acute pediatric malnutrition elicits a regulated shift toward a non-inflammatory form of immune competence [2,9,29,30], the antithesis of the unregulated immunological attrition and exhaustion that is widely presumed. Responsiveness of a dendritic cell population to Flt3L, therefore, is predicted by the Tolerance Model even in advanced stages of malnutrition, and this finding (Tables 3–5) adds incrementally to the evidence base for the model - all the more emphatically in view of the metabolic network required to support dendritic cell development [64]. To date, however, the Tolerance Model has been based on reports in which the primary endpoint is an immunological outcome of limited scope, e.g. a blood index or the size of an effector cell compartment [2,9,29,30]. Consequently, the most significant outcome of this investigation is evidence that immunological plasticity in the face of escalating malnutrition extends to an adaptive response that reflects systemic immune character. This evidence provides new support of fundamental substance for the Tolerance Model and leads to questions centered on mechanisms and prospects for managing infectious disease resistance.

Blood cytokines represent spillover from sites of extravascular production and their concentrations can be regarded as reflective, although probably not representative, of concentrations at sites of cytokine action [2]. Consequently, when the networking characteristics of cytokine mediators are recognized [2,34], their blood concentration profile provides an index of immunological regulatory disposition. In synchrony with the Tolerance Model, both human- and animal-based studies including the experimental system used herein [2,52,65,66], combine to reveal a dramatic shift toward a non-inflammatory blood cytokine profile that is sustained into advanced stages of acute prepubescent malnutrition. In turn, the Tolerance Model necessarily posits conservation of the cytokine backdrop required to uphold a non-inflammatory form of immune competence; hence, it is of interest that the blood concentration of Flt3L was sustained despite advanced weight loss in the present investigation (Table 2). This finding is consistent with the predictions of the Tolerance Model and is the latest addition to our growing knowledge of the blood cytokine profile supported during acute pre-pubescent deficits of protein and energy. Comparison of the blood concentrations of Flt3L sustained in well-nourished and malnourished animals given exogenous cytokine (Tables 1 and 2, respectively) suggests that, in contrast with several anti-inflammatory cytokines [2,9], a low rate of turnover is sufficient to preserve blood levels of Flt3L in the form of acute weanling malnutrition examined here.

In sum, this report provides new evidence of the need, highlighted previously [2,9,29,30], for a paradigm shift so as to view malnutrition-associated inflammatory immune depression through the lens of the Tolerance Model and regulated immunological adaptation. From the standpoint of potential applications, proof-of-concept evidence suggesting plasticity in systemic adaptive inflammatory competence provides a basis for optimism with respect to the management of debilitated patients. It is of particular interest that the experimental system used in this investigation represents a window of developmental vulnerability corresponding, as outlined elsewhere [24], to the first five postnatal years of human immunological ontogeny. Moreover, the dendritic cell compartment is emerging as a portal to inflammatory immune restoration in the face of acute deficits of protein and energy. A dendritic cell-centric model connects easily with the nascent clinical modality of cellular therapeutics [67], and the potency of Flt3L in the present investigation dovetails with a recent report [21] promoting the therapeutic potential of this cytokine. Flt3L merits attention as both an adjunctive tool and an independent therapeutic agent for the management of infection in acute pre-pubescent malnutrition.

Author contributions

The authors jointly formulated the research questions, designed the investigations, performed the statistical analyses and wrote the manuscript. L.M.H. performed the experiments.

Conflict of interest

The authors have no conflict of interest to disclose.

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References

[1] R.E. Black, C.G. Victora, S.P. Walker, Z.A. Bhutta, P. Christian, M. de Onis, et al., Maternal and child undernutrition and overweight in low-income and middle-income countries, Lancet 382 (9890) (2013) 427–451.
[2] B. Woodward, J.M. Monk, The tolerance model: A non-inflammatory form of immune competence prevails in acute pre-pubescent malnutrition, in: J.B. Knudsen (Ed.), Malnutrition: Risk Factors, Health Effects and Prevention, Nova Science Publishers, Hauppauge, New York, 2012, pp. 73–90.
[3] M.J.H. Rytter, L. Kolte, A. Briend, H. Friis, V.B. Christensen, The immune system in children with malnutrition – a systematic review, PLOS One 9 (2014) e105017.
[4] S.M. Hughes, B. Amadi, M. Mwiya, H. Nkamba, A. Tomkins, D. Goldblatt, Dendritic cell anergy results from endotoxemia in severe malnutrition, J. Immunol. 183 (2009) 2818–2826.
[5] M.K. Ibrahim, M. Zambruni, C.L. Melby, P.C. Melby, Impact of childhood malnutrition on host defence and infection, Clin. Microbiol. Rev. 30 (2017) 919–971.
[6] B. Woodward, The effect of protein-energy malnutrition on immune competence, Vevey/Lippincott Williams & Wilkins, Philadelphia, 2001, pp. 89–116.
[7] G.H. Brundtland, Nutrition and infection: Malnutrition and mortality in public health, Nutr. Rev. 58 (2000) S1–S4.
[8] L. Hillyer, C. Whitley, A. Olver, M. Webster, T. Steevels, B. Woodward, Adoptively transferred dendritic cells restore primary cell-mediated inflammatory competence to acutely malnourished weanling mice, Am. J. Pathol. 172 (2008) 378–385.
[9] B. Woodward, Fidelity in animal modeling: prerequisite for a mechanistic research front relevant to the inflammatory incompetence of acute pediatric malnutrition, Int. J. Molec. Sci. 17 (2016) 541, http://dx.doi.org/10.3390/ijms17040541.
[10] B. Woodward, Caveat regarding dendritic cell numbers and functions in murine protein-energy malnutrition, J. Nutr. 137 (2007) 2166.
[11] H.D. Hickman, L. Li, G.V. Reynoso, E.J. Rubin, C.N. Skon, J.W. Mays, et al., Chemokines control naive CD8$^+$ T cell selection of optimal lymph node antigen presenting cells, J. Exp. Med. 208 (2011) 2511–2524.
[12] G.T. Beltz, S.L. Nutt, Transcriptional programming of the dendritic cell network, Nat. Rev. Immunol. 12 (2012) 101–113.
[13] R.M. Kedl, W.A. Rees, D.A. Hildeman, B. Schaefer, T. Mitchell, J. Kappler, et al., T cells compete for access to antigen-bearing antigen-presenting cells, J. Exp. Med. 192 (2000) 1105–1113.
[14] C. Waskow, K. Liu, G. Darrasse-Jeze, P. Guermonprez, F. Ginhoux, M. Merad, et al., The receptor tyrosine kinase Flt3 is required for dendritic cell development in peripheral lymphoid tissues, Nat. Immunol. 9 (2008) 676–683.
[15] M. Merad, M.G. Manz, Dendritic cell homeostasis, Blood 113 (2009) 3418–3427.
[16] E. Maraskovsky, K. Brasel, M. Teepe, E.R. Roux, S.D. Lyman, K. Shortman, et al., Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell subpopulations identified, J. Exp. Med. 184 (1996) 1953–1962.
[17] E. Maraskovsky, E. Daro, E. Roux, M. Teepe, C.R. Maliszewski, J. Hoek, et al., In vivo generation of human dendritic cell subsets by Flt3 ligand, Blood 96 (2000) 878–884.
[18] B. Pulendran, J. Banchereau, S. Burkeholder, E. Kraus, E. Guinet, C. Chalouni, et al., Flt3-ligand and granulocyte colony-stimulating factor mobilize distinct human dendritic cell subsets in vivo, J. Immunol. 165 (2000) 566–572.
[19] M. O'Keeffe, H. Hochrein, D. Vremec, J. Pooley, R. Evans, S. Woulfe, et al., Effects of administration of progenipoietin 1, Flt-3 ligand, granulocyte colony-stimulating factor, and pegylated granulocyte-macrophage colony-stimulating factor on dendritic cell subsets in mice, Blood 99 (2002) 2122–2130.
[20] T. Teshima, P. Reddy, K.P. Lowler, K. KuKuruga, C. Liu, K.R. Cooke, et al., Flt3 ligand therapy for recipients of allogeneic bone marrow transplants expands host CD8α$^+$ dendritic cells and reduces experimental acute graft-versus-host disease, Blood 99 (2002) 1825–1832.
[21] N. Anandasabapathy, R. Feder, S. Mollah, S.-W. Tse, M.P. Longhi, S. Mehandru, et al., Classical Flt3L-dependent dendritic cells control immunity to protein vaccine, J. Exp. Med. 211 (2014) 1875–1891.
[22] S. Vollstedt, M. Franchini, H.P. Hefti, B. Odermatt, M. O'Keeffe, G. Alber, et al., Flt3 ligand-treated neonatal mice have increased innate immunity against intracellular pathogens and efficiently control virus infections, J. Exp. Med. 197 (2003) 575–584.
[23] S. Vollstedt, M. O'Keeffe, B. Odermatt, R. Beat, B. Glanzmann, M. Riesen, et al., Treatment of neonatal mice with Flt3 ligand leads to changes in dendritic cell subpopulations associated with enhanced IL-12 and IFN-α production, Eur. J. Immunol. 34 (2004) 1849–1860.
[24] F. Veru, D.P. Laplante, G. Luheshi, S. King, Prenatal maternal stress exposure and immune function in the offspring, Stress 17 (2014) 133–148.
[25] M.D. Drabik, F.C. Schnure, K.T. Mok, L.L. Moldawer, C.A. Dinarello, G.L. Blackburn, Effect of protein depletion and short-term parenteral refeeding on the host response to interleukin 1 administration, J. Lab. Clin. Med. 109 (1987) 509–516.
[26] Y. Honda, K. Takahashi, M. Naito, S. Fujiyama, The role of macrophage colony-stimulating factor in the differentiation and proliferation of Kupffer cells in the liver of protein-deprived mice, Lab Invest. 72 (1995) 696–706.
[27] A.D.K. Hill, H. Naama, J. Shou, S.E. Calvano, J.M. Daly, Antimicrobial effects of granulocyte-macrophage colony-stimulating factor in protein-energy malnutrition, Arch. Surg. 130 (1995) 1273–1278.
[28] M.A.R. Vinolo, A.R. Crisma, K. Nakajima, M.M. Rogero, R.A. Fock, P. Borelli, Malnourished mice display an impaired hematologic response to granulocyte colony-stimulating factor administration, Nutr. Res. 28 (2008) 791–797.
[29] J.M. Monk, C.L. Richard, B. Woodward, A non-inflammatory form of immune competence prevails in acute pre-pubescent malnutrition: New evidence based on critical mRNA transcripts in the mouse, Br. J. Nutr. 107 (2012) 1249–1253.
[30] J.M. Monk, T.A.M. Steevels, L.M. Hillyer, B. Woodward, Constitutive, but not challenge-induced, interleukin-10 production is robust in acute pre-pubescent protein and energy deficits: New support for the tolerance hypothesis of malnutrition-associated immune depression based on cytokine production in vivo, Int. J. Environ. Res. Public Health 8 (2011) 117–135.
[31] S.M. Filteau, B. Woodward, 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. 112 (1982) 1974–1977.
[32] B.D. Woodward, K.D. Bezanson, L.M. Hillyer, W.-H. Lee, The CD45RA$^+$ (quiescent) cellular phenotype is overabundant relative to the CD45RA phenotype within the involuted splenic T cell population of weanling mice subjected to wasting protein-energy malnutrition, J. Nutr. 125 (1995) 2471–2482.
[33] S. Reagan-Shaw, M. Nihal, N. Ahmad, Dose translation from animal to human studies revisited, FASEB J. 22 (2007) 659–661.
[34] L.M. Hillyer, B. Woodward, Interleukin-10 concentration determined by sandwich enzyme-linked immunosorbent assay is unrepresentative of bioactivity in murine blood, Am. J. Physiol. Regul. Integr. Comp. Physiol. 285 (2003) R1514–R1519.
[35] K. Shipp, B. Woodward, A simple exsanguination method that minimizes acute, pre-anesthesia stress in the mouse: evidence based on serum corticosterone concentrations, Contemp. Top. Lab Anim. Sci. 37 (1998) 73–77.
[36] P.J. Murray, T.A. Wynn, Protective and pathogenic functions of macrophage subsets, Nat. Rev. Immunol. 11 (2011) 723–737.
[37] S. Jung, D. Unutmaz, P. Wong, G. Sano, K. De los Santos, T. Sparwasser, et al., In vivo depletion of CD11c$^+$ dendritic cells abrogates priming of CD8$^+$ T cells by exogenous cell-associated antigens, Immunity 17 (2002) 211–220.
[38] N. Anandasabapathy, G.D. Victora, M. Meredith, R. Feder, B. Dong, C. Kluger, et al., Flt3L controls the development of radiosensitive dendritic cells in the meninges and choroid plexus of the steady-state mouse brain, J. Exp. Med. 208 (2011) 1695–1705.
[39] E.L. Gautier, T. Shay, J. Miller, M. Greter, C. Jakubzik, S. Ivanov, et al., Gene-expression profiles and transcriptional regulatory pathways that underlie the identity and diversity of mouse tissue macrophages, Nat. Immunol. 13 (2012) 1118–1130.
[40] A.T. Kamath, J. Pooley, M.A. O'Keeffe, D. Vremec, Y. Zhan, A.M. Lew, et al., The development, maturation and turnover rate of mouse spleen dendritic cells, J. Immunol. 165 (2000) 6762–6770.
[41] N.S. Wilson, L.J. Young, F. Kupresanin, S.H. Naik, D. Vremec, W.R. Heath, et al., Normal proportion and expression of maturation markers in migratory dendritic cells in the absence of germs or Toll-like receptor signaling, Immunol. Cell Biol. 86 (2008) 200–205.
[42] M. Lechmann, N. Shuman, A. Wakeham, T.W. Mak, The CD83 reporter mouse elucidates the activity of the CD83 promoter in B, T and dendritic cell populations *in vivo*, Proc. Nat. Acad. Sci. USA 105 (2008) 11887–11892.
[43] R.R. Hardy, K. Hayakawa, B cell development pathways, Annu. Rev. Immunol. 19 (2001) 595–621.
[44] J.W.M. Visser, G.J. van den Engh, D.W. van Bekkum, Light scattering properties of murine hemopoietic cells, Blood Cells 6 (1980) 391–407.
[45] B. Obermaier, M. Dauer, J. Herten, K. Schad, S. Endres, A. Eigler, Development of a new protocol for 2-day generation of mature dendritic cells from human monocytes, Blood Proc. Online 5 (2003) 197–203.
[46] Mathematics Education Centre, Loughborough University, UK, (2006) Statstutor http://www.statstutor.ac.uk/topics/sample-size-and-statistical-power (accessed October 2016).
[47] J.E. Konyer, L.M. Hillyer, B. Woodward, 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. 23 (2003) 921–931.
[48] B.D. Woodward, J.W. Woods, D. Crouch, Direct evidence that primary acquired cell-mediated immunity is less resistant than primary thymus-dependent humoral immunity to the depressive influence of wasting protein-energy malnutrition in the weanling mouse, Am. J. Clin. Nutr. 55 (1992) 1180–1185.
[49] S.N. Robinson, J.M. Chavez, V.M. Pisarev, R.L. Mosley, G.J. Rosenthal, J.M. Blonder, et al., Delivery of Flt3 ligand (Flt3L) using a poloxamer-based formulation increases biological activity in mice, Bone Marrow Transplant. 31 (2003) 361–369.
[50] K. Liu, A. Charalambons, R.M. Steinman, Some biological features of dendritic cells in the mouse, 2nd ed., Academic Press, Amsterdam, Boston, 2007, pp. 135–154.
[51] C. Asselin-Paturel, G. Brizard, J.-J. Pin, F. Briere, G. Trinchieri, Mouse strain differences in plasmacytoid dendritic cell frequency and function revealed by a novel monoclonal antibody, J. Immunol. 171 (2003) 6466–6477.
[52] L.M. Hillyer, H.E. Maliwichi, B. Woodward, Blood serum interferon-γ bioactivity is low in weanling mice subjected to acute deficits of energy or both protein and energy, Br. J. Nutr. 97 (2007) 528–534.
[53] T.A.M. Steevels, L.M. Hillyer, J.M. Monk, M.E. Fisher, B.D. Woodward, 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, J. Nutr. Biochem. 21 (2010) 504–511.
[54] X. Zhang, L.M. Hillyer, B.D. Woodward, The capacity of non-inflammatory (steady-state) dendritic cells to present antigen in the primary response is preserved in acutely protein- or energy-deficient weanling mice, J. Nutr. 132 (2002) 2748–2756.
[55] J.W. Woods, B.D. Woodward, Enhancement of primary systemic acquired immunity by exogenous triiodothyronine in the wasted, protein-energy malnourished weanling mouse, J. Nutr. 121 (1991) 1425–1432.
[56] Z.C. Neal, P.M. Sondel, M.K. Bates, S.D. Gillies, H. Herweijer, Flt3-L gene therapy enhances immunocytokine-mediated antitumor effects and induces long-term memory, Cancer Immunol. Immunother. 56 (2007) 1765–1774.
[57] D.J. Manfra, C. Shu-Cheng, K.K. Jensen, J.S. Fine, M.T. Wiekowski, S.A. Lira, Conditional expression of murine Flt3 ligand leads to expansion of multiple dendritic cell subsets in peripheral blood and tissues of transgenic mice, J. Immunol. 170 (2003) 2843–2852.
[58] C.A. Jolly, G. Fernandes, Protein-energy malnutrition and infectious disease, Humana Press, Totowa, NJ, 2000, pp. 195–202.
[59] B. Hurtrel, M.-A. Maire, M. Hurtrel, P.H. Lagrange, Different time course patterns of local expression of delayed-type hypersensitivity to sheep red blood cells in mice, Cell Immunol. 142 (1992) 252–263.
[60] P.W. Askenase, B. Hayden, R.K. Gershon, Evanescent delayed-type hypersensitivity: Mediation by effector cells with a short life span, J. Immunol. 119 (1977) 1830–1835.
[61] K. Shortman, S.H. Naik, Steady-state and inflammatory dendritic-cell development, Nat. Rev. Immunol. 7 (2007) 19–30.
[62] T. Niiya, S.M.F. Akbar, O. Yoshida, T. Miyake, B. Matsuura, H. Murakami, et al., Impaired dendritic cell function resulting from chronic undernutrition disrupts the antigen-specific immune response in mice, J. Nutr. 137 (2007) 671–675.
[63] S. Kreiter, M. Diken, A. Selmi, J. Diekmann, S. Attig, Y. Husemann, et al., Flt3 ligand enhances the cancer therapeutic potency of naked RNA vaccines, Cancer Res. 71 (2011) 6132–6142.
[64] L.A.J. O'Neill, E.J. Pearce, Immunometabolism governs dendritic cell and macrophage function, J. Exp. Med. 213 (2016) 15–23.
[65] I. Hagel, N.R. Lynch, M.C. Di Prisco, J. Sanchez, M. Perez, Nutritional status and the IgE response against Ascaris lumbricoides in children from a tropical slum, Trans. Roy. Soc. Trop. Med. Hyg. 89 (1995) 562–565.
[66] C. Gonzalez-Torres, H. Gonzalez-Martinez, A. Miliar, O. Najera, J. Graniel, V. Firo, et al., Effect of malnutrition on the expression of cytokines involved in Th1 cell differentiation, Nutrients 5 (2013) 579–593.
[67] A.L. Fletcher, J.S. Elman, J. Astarita, R. Murray, N. Saeidi, J. D'Rozario, et al., Lymph node fibroblastic reticular cell transplants show robust therapeutic efficacy in high-mortality murine sepsis, Sci. Translat. Med. 6 (2014) 249ra109.