Maternal Perinatal Undernutrition Attenuates T-Cell Function in Adult Male Rat Offspring

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Key Words

Maternal malnutrition • Offspring • T cells • Polyfunction • Cytokines

cytokine production as compared with control offspring. Conclusions. Taken together, these findings suggest that early undernutrition, particularly during the prenatal and lactation periods, attenuates T cell survival and the immune response.

Abstract

Objective. We investigated the impact of maternal food restriction during gestation and lactation on the number and function of peripheral T cells in adult male offspring. **Study design.** Pregnant rats either received *ad libitum* (control) feed or were 50% food restricted (FR) from day zero of gestation to delivery and during the lactation period. After weaning, pups from control and FR mother rats were fed normally until they reached 8 weeks of age. At 8 weeks of age, blood parameters, plasma cytokine levels, and T cell number and function were determined in 10 male offspring from each group. **Results.** FR offspring exhibited decreased numbers of total and CD4⁺ and CD8⁺ T lymphocytes and a significant reduction in plasma IL-2 and IL-7 compared with control offspring. In addition, T lymphocytes from FR offspring exhibited a 2-fold decrease in IL-2- and IL-7-mediated AKT and STAT5 phosphorylation, altered actin polymerization, and diminished superantigen-mediated proliferation and

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Introduction

Malnutrition is caused by disturbances in the equilibrium between dietary intake and nutrient needs. Perinatal malnutrition has long-term effects on postnatal development. This concept, which was advanced by Barker as the “developmental origins of health and disease” hypothesis, is relevant to the postnatal maturation of the immune response [1]. Malnutrition represents a major public health problem and is a substantive underlying factor in over 50% of the 10-11 million deaths occurring in children each year [2]. Acute malnutrition can lead to increased susceptibility to infection and impaired immunity [3]. In severely malnourished patients, both acquired and

innate immune defenses are simultaneously diminished [4]. Malnutrition also depresses the cell-mediated form of immune competence both in children and in relevant rodent models [5]. In this context, investigators have shown that the functional capacities of immune cells decrease in subjects with nutritional deficiencies [6, 7].

Several studies on the effects of malnutrition at the immunological level have been carried out in humans and animal models. These studies indicate that malnutrition decreases T cell function, cytokine production, and the ability of lymphocytes to respond appropriately to cytokines [8]. We have recently reported that T lymphocytes from malnourished infants are short lived and dysfunctional [9]. Although there is a large body of literature addressing the effect of maternal malnutrition on immune responses, the proliferative response of T cells is still poorly defined.

Cytokines are substances that play an important role in coordinating the inflammatory response to various external and internal stimuli. Many studies have described the effect of malnutrition on cytokine expression [10, 11]. CD4⁺ and CD8⁺ T cells from malnourished children show increased levels of IL-4 and IL-10 cytokines and decreased levels of IL-2 and IFN- γ cytokines compared to cells from well-nourished children [10]. In addition, the production of IL-6 and TNF- α by bone marrow cells has been found to be significantly lower in malnourished animals [12]. One of the main causes of this depressed immunity during undernutrition appears to be altered T cell activation. For example, impaired T cell activation was shown to be clearly associated with deficits in cytokine production in a study of malnourished children who showed reduced IL-2 and IFN- γ production [10].

Cell proliferation after mitogenic stimulation is an important parameter used in the diagnosis of immunodeficiency in clinical laboratory research and in various fields of lymphocyte research. Signal Transducers and Activators of Transcription (STATs) play a central role in the cytokine dependent survival and proliferation of hematopoietic cells [13]. The PI3-kinase/AKT pathway also plays an important role in cytokine-induced cell survival and/or proliferation. Recently, Hand et al. reported that the viability of effector and memory CD8 T cells depends on properly balanced PI3K/AKT signaling and on the maintenance of STAT5 signaling [14]. Lymphocyte apoptosis has been described in peripheral blood and lymphatic organs during infection and malnutrition [15]. It has also been reported that enhanced apoptosis in the peripheral blood leukocytes of protein energy malnutrition patients may be a marker of increased infection and

immune disturbances [16]. T cell activation, division, adhesion, and migration involve reorganization of the actin cytoskeleton. Cellular mobility also requires complex molecular interactions, both between chemokines and T cell chemokine receptors and between adhesion molecules and their ligands on other cells or in the extracellular matrix. Such interactions activate signaling pathways that trigger dynamic cytoskeletal rearrangements, which enable the lymphocyte to adhere to and migrate over its substrates, e.g., antigen presenting cells (APC), and to transmigrate through the endothelium [17, 18].

The molecular mechanism of altered T cell function in maternal perinatal undernutrition is not yet understood. Therefore, in the present study we investigated the impact of maternal malnutrition during pregnancy on T cell activation, proliferation and cytokine production in male rat offspring.

Materials and Methods

Animals and experimental design

Sexually mature female Sprague-Dawley laboratory rats weighing 180–220 g each were obtained from the Central Animal House of the Faculty of Pharmacy at King Saud University. All animal procedures were in accordance with the standards set forth in the guidelines for the care and use of experimental animals by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and the National Institutes of Health (NIH) protocol. The study protocol was approved by the Animal Ethics Committee of the Zoology Department, College of Science, King Saud University. All animals were allowed to acclimatize in metal cages inside a well-ventilated room for 2 weeks prior to the experiment. Animals were maintained under standard laboratory conditions (temperature 23°C, relative humidity 60–70% and a 12-hour light/dark cycle) and were fed a diet of standard commercial pellets and water.

Feeding protocol

Timed mating was carried out in age-matched (12- to 16-wk-old) female and male rats. The presence of a vaginal plug the following morning was taken as evidence of mating, and that day was counted as day 1 of pregnancy. After confirming that mating had occurred, the rats were housed individually in standard rat cages. Female rats were randomly divided into 2 groups: a control group and a food-restricted group (FR). Rats in the NR group were fed a standard commercial rat diet containing protein (minimum 22.0%), carbohydrates (maximum, 54.0%), fat (minimum, 4.5%), cellulose (maximum, 8.0%), minerals (maximum, 10.0%), water (maximum, 12.5%), and vitamins. Rats in the FR group were fed the same diet at 50% of the NR intake (as determined by the amount of food consumed by the control group) from d 1 of pregnancy until weaning. All rats were fed

daily, in the morning, and consumption was determined 24 h later. The body weight of dams was determined daily during pregnancy, and the body weight of pups was determined at birth. FR pups differed from NR pups only in their prenatal dietary experience. To prevent variation in neonatal growth related to the unavailability of milk during suckling, litter size was standardized to 8 pups (4 males and 4 females whenever possible). After weaning, the pups of the two groups were fed the same diet. Offspring body weight and food intake were followed until the age of 2 months. At 8-9 weeks of age, 10 male offspring from different dams in each group were used for experiments.

Blood samples, plasma, PBMCs and CD3⁺ T cell isolation

After overnight fasting, 10 male offspring from each group were anesthetized with pentobarbital (60 mg/kg body weight). The abdominal cavity was opened, and blood was collected via the abdominal aorta. Plasma was obtained by low-speed centrifugation (1000 x g for 20 min) and immediately stored at -80°C for later examination. After plasma isolation, peripheral blood mononuclear cells (PBMCs) were isolated using the Ficoll gradient method. Freshly isolated PBMCs were cultured in RPMI 1640 supplemented with 10% FCS and Hepes (R-10 medium) for at least 4 hours prior to the start of the experiments. In some experiments, CD3⁺ T cells were purified from the freshly isolated PBMCs using a MagCollect Rat CD3⁺ T cell isolation kit (R&D Systems Europe Ltd., Abingdon, UK) according to the manufacturer's protocol. The final purity was 92–95% (as assessed by flow cytometry).

Blood cell count and measurements

Immediately after blood withdrawal, values of hematological parameters were measured using a dedicated animal hematology autoanalyzer (Scil Vet abc Hämatologie™, Scil Animal Care Company GmbH, Viernheim, Germany). These included hemoglobin concentration (g/dl), hematocrit value (%), and absolute number ($\times 10^3/\text{mm}^3$) of red blood cells (RBCs), white blood cells (WBCs), lymphocytes, and neutrophils.

Determination of plasma cytokine levels

Determination of the plasma cytokine profiles was performed using samples that had been stored at -80°C. Plasma cytokine (IL-1 β , IL-2, IL-4, IL-6, IL-7, IL-10 and TNF- α) levels were determined by ELISA using a Bio-Plex Rat cytokine assay kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions.

Flow cytometric analysis of T cell subsets and surface antigens

PBMCs were stained using the following PE (phycoerythrin)- and FITC (fluorescein isothiocyanate)-labeled monoclonal antibodies (mAbs): W3/25 (CD4), OX-8 (CD8), CD3-APC and isotype controls. All antibodies were purchased from Serotec (Raleigh, NC). Flow cytometry data were collected using a BD FACSCalibur flow cytometer and expressed as the percentage of T cells in the total PBMCs (determined according to FSC/SSC).

Western blot analysis

Purified CD3⁺ T cells were resuspended at a density of 1×10^7 cells/ml in pre-warmed RPMI 1640 without FCS and stimulated for 5 min at 37°C with IL-2 (20 IU/ml) or IL-7 (1 ng/ml). Lysates were prepared as previously described [19]. Equal amounts of total cellular protein were subjected to SDS-PAGE and blotted onto a nitrocellulose membrane (MilliPore, Bedford, MA, USA). Primary antibodies recognizing phospho-AKT (S473) and AKT were purchased from MilliPore (Bedford, MA, USA). The STAT5 antibody that detects both STAT5a and STAT5b was from Santa Cruz Biotechnology (Santa Cruz, CA), and the phospho-STAT5 antibody directed against the tyrosine-phosphorylated forms of both STAT5a and STAT5b was from Upstate Biotechnology (Lake Placid, NY). All primary antibodies were diluted 1:1000. After incubation with secondary antibodies conjugated to HRP (rabbit anti-mouse [DAKO] or goat anti-rabbit [Santa Cruz Biotechnology]), the antigens were visualized using chemiluminescence (ECL, Supersignal West Pico chemiluminescent substrate, Perbio, Bezons, France) and acquired with X-ray film (Amersham Biosciences, France). The ECL signal was recorded on ECL hyperfilm. To quantify band intensities, films were scanned, saved as TIFF files and analyzed with NIH ImageJ software.

F-actin polymerization assay

Intracellular F-actin polymerization was assessed as previously described [20]. PBMCs from each group ($8 \times 10^6/\text{ml}$) were incubated in HEPES-buffered RPMI 1640 at 37°C, with or without anti-CD3/CD28. At the indicated times, cells ($100 \mu\text{l}$) were added to $400 \mu\text{l}$ of assay buffer containing 4×10^{-7} M FITC-labeled phalloidin, 0.5 mg/ml L- α -lysophosphatidylcholine (both from Sigma-Aldrich) and 4.5% formaldehyde in PBS. Fixed cells were analyzed by flow cytometry, and the mean fluorescence intensity (MFI) was determined for each sample. The percent change in MFI was calculated for each sample at each time point as follows: $[1 - (\text{MFI before addition of anti-CD3+CD28} / \text{MFI after addition of anti-CD3+CD28})] \times 100$.

CFSE proliferation assays

PBMCs were isolated from heparinized blood using the Ficoll gradient method as previously described [21]. PBMCs were resuspended at 20×10^6 cells/ml in 1X phosphate-buffered saline (PBS) and stained with 0.63 mM carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR) for 8 min at room temperature. The reaction was stopped by the addition of FBS, and the cells were washed three times in PBS and then resuspended at 1×10^6 cells/ml in prewarmed R-10 medium. CFSE-labeled cells were stimulated for 6 days with or without staphylococcal enterotoxin B (SEB) (5 ng/ml) at 37°C and 5% CO₂. On day 6, the cells were stained to assess surface antigens using anti-CD3-PE.

Intracellular cytokine staining (ICS) assay

PBMCs ($2 \times 10^6/\text{ml}$ R-10) were stimulated with SEB (5 ng/ml) for 1 hour at 37°C and 5% CO₂ in 5 ml snap-cap polypropylene tubes set at an angle of 5°. Following one hour of either stimulation or rest, $10 \mu\text{g}/\text{ml}$ of brefeldin A (Sigma-Aldrich, France) and $6 \mu\text{g}/\text{ml}$ of monensin sodium salt (Sigma-

	WBCs ($\times 10^3/\text{mm}^3$)	Lymphocytes ($\times 10^3/\text{mm}^3$)	Neutrophils ($\times 10^3/\text{mm}^3$)	RBCs ($\times 10^3/\text{mm}^3$)	HB (g/dl)	Hematocrit value (%)
Control Offspring	9.91 $\pm$ 0.6	5.88 $\pm$ 0.44	4.2 $\pm$ 0.4	5.5 $\pm$ 0.2	13.3 $\pm$ 0.9	34 $\pm$ 1.9
FR Offspring	7.1 $\pm$ 0.45 *p<0.05	3.75 $\pm$ 0.35 *p<0.05	4 $\pm$ 0.38	5.35 $\pm$ 0.42	13.1 $\pm$ 0.8	33.77 $\pm$ 2.4

Table 1. Blood parameters in control and FR offspring. Results are expressed as means $\pm$ SEM. *P < 0.05.

Aldrich, France) were added, and cells were incubated for a total of 12 hrs. Cells were then washed with FACS buffer, stained for 30 min at 4°C CD3-APC, and permeabilized using BD Cytofix/Cytoperm solution (BD Bioscience, France). Intracellular staining with anti-IL-2-FITC (Abcam) and anti-IFN- γ -PE (BD Biosciences) was performed for 30 minutes, and the cells were washed twice in BD Perm/Wash buffer (BD Biosciences, France) and fixed in 200 μ l of FACS fix buffer. Polyfunctional data were exported using Boolean gates in FlowJo and further analyzed using PESTLE software (version 1.5.4) and SPICE software (version 4.1.6) obtained from M. Roederer, National Institutes of Health, Bethesda, MD. For analysis, cells were gated on viable CD3⁺ T cells. The percent specific expression was calculated as a background-adjusted function in the presence or absence of CD3/CD28 stimulation. Polyfunctional T cells were defined as viable CD3⁺ T cells that produce IFN- γ and IL-2 simultaneously (IFN- γ + IL-2), in contrast to monofunctional T cells, which produce either IL-2 or IFN- γ alone.

Statistical analysis

Data were expressed as means $\pm$ SEM (standard error of the mean). Statistical differences between groups were analyzed by one-way analysis of variance (ANOVA) using SPSS software version 17. Differences were considered statistically significant at P < 0.05.

Results

Decreased T cell numbers in food restricted offspring

We first compared blood parameters in the control and FR offspring. We observed a significant decrease in the total number of white blood cells and lymphocytes in the FR offspring compared with the control offspring (Table 1). Nevertheless, the total numbers of red blood cells and neutrophils, as well as the hemoglobin and hematocrit values, were not significantly different between the two groups of offspring. To further investigate the phenotypic changes in the T cell populations, we used monoclonal antibodies targeting CD3 (pan-T cell) and CD4 and CD8 antibodies to discriminate between helper (CD4⁺) and cytotoxic (CD8⁺) T cell populations using flow cytometric

analysis. The percentage of CD4⁺ and CD8⁺ T cells was clearly reduced in the FR offspring as compared with the control offspring (Fig. 1D, C). In contrast, CD4/CD8 double positive T cell population exhibited a 4-fold increase in FR offspring as compared with the control offspring. Combined data from ten offspring in each group revealed that the percentage of CD4⁺ T cells was significantly (*P<0.05) reduced from 71 $\pm$ 4.6 in control offspring to 57 $\pm$ 5.1 in FR offspring (Fig. 1E). Similarly, the percentage of CD8⁺ T cells was significantly (*P<0.05) reduced from 25 $\pm$ 2.7 in control offspring to 15 $\pm$ 1.4 in FR offspring.

Decreased plasma cytokine levels of IL-2 and IL-7 in food restricted offspring

Plasma cytokine levels of IL-1 β , IL-2, IL-4, IL-6, IL-7, IL-10 and TNF- α were evaluated in the offspring of the two groups. The levels of IL-2 and IL-7 were significantly (*P<0.05) reduced from 35 $\pm$ 3.3 and 47 $\pm$ 4.4 pg/ml, respectively, in control offspring to 18 $\pm$ 2.1 and 27 $\pm$ 2.4, respectively, in FR offspring (n=10) (Fig. 2). However, the plasma levels of IL-1 β , IL-4, IL-6, IL-10 and TNF- α were not significantly different between the two groups.

Decreased IL-2- and IL-7-mediated phosphorylation of AKT and STAT5 in the T cells of food restricted offspring

Decreased plasma levels of IL-2 and IL-7 or altered IL-2/IL-7 signaling could be the cause of the observed decrease in T cell responsiveness. Therefore, we investigated whether IL-2 and IL-7 signaling, which is important for T-cell survival and long term responsiveness, was impaired in FR offspring. We focused on the phosphorylation of AKT and STAT5, which are the main signaling pathways downstream of IL-2 and IL-7 in T cells. We found that IL-2 and IL-7 induced different patterns of AKT and STAT5 phosphorylation in control and FR offspring, as shown by Western blot (Fig. 3). Combined data from five separate experiments revealed that when the levels of phosphorylated AKT and STAT5 (P-AKT and P-STAT5)

Fig. 1. Phenotypic analysis of blood T cell populations in offspring groups. Freshly isolated PBMCs were cultured in R-10 culture media at 37°C for 4 hours before staining with antibodies. (A) After gating 25000 events per treatment, total lymphocytes were gated according to their FSC/SSC scatter. (B) T cell levels measured using anti-CD3 antibody. (C and D) Representative dot-plots of CD4⁺ and CD8⁺ T cells from one representative experiment are shown for control (C) and FR (D) offspring. The experiment was repeated with samples from 10 different rats from control offspring (closed black bars) and FR offspring (closed gray bars) groups, and results are expressed as the mean percentage of viable T cell populations $\pm$ SEM (n=10), $P < 0.05$.

were normalized to the total amount of the relevant total protein (T-AKT and T-STAT5), phosphorylation of AKT and STAT5 increased significantly following stimulation with IL-2 and IL-7 (10 and 12 fold, respectively) ($*P < 0.02$) in control offspring (Fig. 3A, C). In contrast, in FR offspring, the ability of IL-2 and IL-7 to mediate phosphorylation of AKT and STAT5 was significantly lower ($*P < 0.05$) (5- to 6-fold, respectively) (Fig. 3B, D). When the two groups were compared, the ability of IL-2 and IL-7 to mediate phosphorylation of AKT and STAT5 was significantly ($*P < 0.02$) decreased by 2 fold in FR offspring compared with control offspring.

Fig. 2. Plasma cytokine profiles in the two offspring groups. Plasma cytokine levels were determined for the offspring of each group (n=10). Data are presented as the mean level of each cytokine $\pm$ SEM, $P < 0.05$.

Fig. 3. IL-2- and IL-7-mediated phosphorylation of AKT and STAT5 in T cells is altered in FR offspring. Phosphorylation of AKT (pAKT) and STAT5 (pSTAT5), the main signaling pathway downstream of IL-2 and IL-7, was investigated using Western blotting analysis. CD3⁺ T cells were stimulated with medium (0), IL-2, or IL-7 and then lysed and analyzed by Western blotting. The results of one representative experiment of five are shown. AKT and STAT5 phosphorylation (P-AKT and P-STAT5) was normalized against total relevant protein levels (T-AKT and T-STAT5) on stripped blots. Black bars (A and C) show the results for five control offspring rats, expressed as the mean $\pm$ SEM of the normalized phosphorylation values of AKT and STAT5. Gray bars (B and D) show the results for five FR offspring rats, expressed as the mean $\pm$ SEM of the normalized phosphorylation values of AKT and STAT5.

TCR-mediated F-actin polymerization and T-cell responsiveness is diminished in food restricted offspring

Actin and microtubules provide the dynamic cellular framework needed to orchestrate and ultimately control T cell activation, immunological synapse formation, and proliferation. Therefore, actin polymerization upon TCR/costimulation was monitored in the T cells of control and food-restricted offspring. PBMCs were stimulated every 15 sec with anti-CD3/CD28 mAbs, and the degree of F-actin polymerization was determined by flow cytometry. In the control offspring, the percentage of cells positive for F-actin polymerization was high: 91 ± 6.7 , 76 ± 5.7 , 40 ± 4.1 , and 12 ± 1.8 at 15, 30, 45 and 60 sec, respectively (Fig. 4). In contrast, in the FR offspring the percentage of cells positive for F-actin polymerization was significantly (* $P < 0.03$) reduced to 53 ± 5 , 22 ± 2 , 11 ± 1.7 , and 2 ± 0.9 at 15, 30, 45, and 60 sec, respectively ($n = 10$).

Fig. 4. TCR-mediated F-actin polymerization is impaired in FR offspring. F-actin polymerization was measured in response to anti-CD3/CD28 stimulation of PBMCs isolated from control (closed triangles) and FR (open circles) offspring. Results are expressed as the percentage change in MFI ($n = 10$) $\pm$ SEM as described in the *Materials and Methods*.

Fig. 5. Superantigen-induced T cell proliferation is altered in FR offspring. The ability of T cells to proliferate in response to superantigen stimulation was evaluated using the CFSE assay and flow cytometry. Histograms represent the results of the CFSE dilution assay, in which we gated on viable CD3⁺ T cells among PBMCs for control and FR offspring. The mean of the left histograms represents the percentage of CFSE-low (proliferating cells), while the mean of the right histograms represents the percentage of CFSE-high (non-proliferating cells) within the T cell population. Histograms from one representative experiment are shown for unstimulated (A) and SEB-stimulated (B) cells from control offspring and for unstimulated (C) and SEB-stimulated (D) cells from FR offspring. (E) Data from nine rats in each group are expressed as the percentage of T cell proliferation $\pm$ SEM in control (black bars) or FR (gray bars) offspring; n=9.

Undernutrition reduces superantigen-mediated T cell proliferation

The inability of a cell to mediate F-actin polymerization in response to TCR stimulation is normally accompanied by a decrease in its proliferative capacity. We therefore monitored T-cell proliferation following SEB stimulation using a CFSE dilution assay.

Fig. 6. FR offspring exhibit decreased polyfunctional T-cell production. The ability of activated T cells to produce cytokines was monitored in the two offspring groups. (A) Analysis of cytokine production by ICS assay following SEB stimulation was performed after gating on viable CD3⁺ T cells. Data are represented as the percentage of specific expression of each individual cytokine $\pm$ SEM, i.e., the percentage of IL-2 (black bars) and IFN- γ (green bars). (B) Polyfunctionality of T cells was monitored in viable CD3⁺ T cells, where red represents two simultaneous functions (IFN- γ + IL-2 production), green represents IFN- γ production only, and black represents IL-2 production only on the pie chart. Numbers on the pie chart represent the percentage of cells in each population.

PBMCs were labeled with CFSE, stimulated with SEB and cultured for 6 days prior to staining with anti-CD3-APC. The plots were gated on lymphocytes according to the forward and side scatter, then on viable cells to exclude dead cells, and finally on CD3⁺ T cells. The percentages of CFSE-low (proliferating) and CFSE-high (non-proliferating) cells within the T cell population are

presented as histograms (Fig. 5). In one representative experiment, we found that the percentage of spontaneous (unstimulated) T cell proliferation was nearly similar in control (2%) and FR offspring (1%) (Fig. 5A, C). The percentage of T cell proliferation following SEB stimulation was reduced from 65% in control offspring to 43% in FR offspring (Fig. 5B, D). Data from nine separate experiments revealed that the percentage of proliferating T cells was significantly ($*P<0.05$) reduced from $73\pm6.3\%$ in control offspring (black bars) to $44\pm4.1\%$ in FR offspring (gray bars), (Fig. 5E).

Undernutrition attenuates the ability of T cells to produce multiple cytokines simultaneously

Next, we analyzed cytokine production to test T-cell function following superantigen stimulation. We found that FR offspring exhibited a decreased percentage of T cells expressing IL-2 or IFN- γ as demonstrated by ICS (Fig. 6A). The percentage of T cells producing IL-2 was significantly decreased from $4\pm0.3\%$ in control offspring to $2.1\pm0.2\%$ in FR offspring ($*P<0.05$). Similarly, the percentage of T cells producing IFN- γ was significantly decreased from $9\pm0.6\%$ in control offspring to $5.2\pm0.4\%$ in FR offspring ($*P<0.05$). The T cells of the FR offspring were primarily monofunctional (IFN- γ producers). Notably, the generation of polyfunctional T cells was robust in the control offspring. Polyfunctional cells constituted 39% of all T cells in the control offspring versus 22% in the FR offspring, respectively (Fig. 6B).

Discussion

Prenatal and early postnatal undernutrition has been linked to deficits in several aspects of cell-mediated immunity, including involution of lymphoid tissues such as the thymus and suppression of antibody responses to vaccination [22]. Furthermore, these deficits carry over into the next generation of offspring, demonstrating a long-term, intergenerational effect of maternal undernutrition on the immune response. Clinical observations and epidemiologic studies lend support to the concept that nutritional deficiencies increase the frequency and severity of infection. In the present study, we demonstrated that restricting maternal nutrition in pregnant rats resulted in a significant decrease in the total number of white blood cells and lymphocytes of FR offspring as well as a clear reduction in the percentage of CD4 $^{+}$ and CD8 $^{+}$ T cells. Similarly,

Collinson et al. have demonstrated that nutritional insult at a critical stage in thymic development may lead to a permanent impairment in T cell immunity because thymic development mainly occurs in utero and in early postnatal life [23]. Furthermore, malnutrition clearly affects hematopoiesis, causing anemia, leucopenia, and a severe reduction in the development of peripheral lymphoid organs [24]. As it has been known that the frequency of CD4/CD8 double-positive T cells in the peripheral blood is increased during viral infections, increased CD4/CD8 double-positive T cells in FR offspring could be due to deficit in T cell immune response or due to infection.

Cytokines encompass a large group of glycoproteins that modulate the activity of individual cells during both physiological and pathological conditions [25]. These polypeptides are synthesized in response to microorganisms and other antigens, and they mediate and regulate immune and inflammatory reactions. In our study, we found that maternal malnutrition significantly decreased IL-2 and IL-7 levels. However, the plasma levels of IL-1 β , IL-4, IL-6, IL-10 and TNF- α were not significantly different between the offspring in the control and food-restricted groups. Similarly, many studies have described changes in the expression of different cytokines, including IL-2 and IFN- γ , during malnutrition [10, 11]. These results suggest that the immune response may be impaired in malnourished patients. Previous findings have indicated that diminished IL-2 production induces CD8 $^{+}$ T cell anergy (i.e., failure to proliferate to antigen), which inhibits autocrine IL-2 production [26]. The present results showed a significant decrease in the levels of circulating IL-7 in FR offspring; this finding may be attributed to T cell dysfunction, as described previously [9].

Several studies have shown that the PI3K/AKT pathway is essential for T cell proliferation, and PI3K pathway-specific inhibitors have been used to show that a late phase of PI3K activity is required for IL-2-induced lymphocyte proliferation [27, 28]. The transcription factor STAT5, which actually refers to the two highly homologous proteins STAT5a and STAT5b, also promotes mitogenesis and antiapoptosis in lymphocytes [29]. In our study, we found that the ability of IL-2 and IL-7 to mediate AKT and STAT5 phosphorylation was significantly decreased by 2-fold in FR offspring compared with control offspring. Similar results were published by Hand et al., who found that effector and memory CD8 $^{+}$ T cell viability depends on properly balanced PI3K/AKT

signaling and on the maintenance of STAT5 signaling [14].

Actin polymerization plays a critical role in many essential processes in activated T lymphocytes, including the regulation of TCR-induced immunological synapse formation and signaling, cell migration, morphogenesis, cellular trafficking, cytokinesis and cell proliferation [30]. In the FR offspring group, the percentage of cells positive for F-actin polymerization was significantly reduced compared with the control group. Defective actin polymerization leads to long-term T cell unresponsiveness or anergy [31]. In addition, cell proliferation after mitogenic stimulation is an important parameter used in the diagnosis of immunodeficiencies in clinical laboratory research and in various fields of lymphocyte research. Our data revealed that diminished F-actin polymerization was accompanied by a significant decrease in the T cell proliferative capacity in FR offspring.

Intracellular cytokine expression is a major response of T cells to stimulation. Analysis of intracellular cytokine expression in T cells by flow cytometry and ICS assay allows for the detection of both phenotype and the ability to express one or more cytokines simultaneously; detecting only plasma cytokine levels, in contrast, gives no information about cytokine-producing

cells. Our data showed that T cells from FR offspring exhibited decreased IL-2 and IFN- γ expression. Moreover, the T cells of FR rats were found to be primarily monofunctional (IFN- γ producers), with polyfunctional cells constituting only 22% of T cells in the FR offspring (versus 39% in the control offspring). Similar observations were made by Badr et al., who attributed the decrease in intracellular cytokine expression to an impaired T cell immune response in malnourished children [9].

In conclusion, the abnormal T cell response following maternal malnutrition can be explained by a diminished proliferative capacity and reduced cytokine production.

Abbreviations

Protein Kinase B (PKB/AKT); Carboxyfluorescein diacetate succinimidyl ester (CFSE); Food restriction (FR); Intracellular cytokine staining (ICS); Interferon gamma (IFN- γ); Interleukin (IL); Peripheral Blood Mononuclear Cells (PBMCs); Signal Transduction and Activator of Transcription 5 (STAT5); Tumor Necrosis Factor alpha (TNF- α).

References

- 1 Barker DJ: The developmental origins of chronic adult disease. *Acta Paediatr Suppl* 2004;93:26-33.
- 2 Collins S: Treating severe acute malnutrition seriously. *Arch Dis Child* 2007;92:453-461.
- 3 Woodward B: Depressed adaptive immune competence in acute protein-energy malnutrition: a regulated pathophysiology controlled by endocrine hormones and cytokines; in Chandra RK (ed): *Nutrition and Immunology in the 21st century*. TSAR Health, Gurgaon/Toronto, 2004, pp 23-38.
- 4 Schaible UE, Kaufmann SHE: Malnutrition and infection: complex mechanisms and global impacts. *PLoS Med* 2007;4:806-812.
- 5 Woodward B: The effect of protein-energy malnutrition on immune competence; in Suskind RM, Tontisirin K (eds): *Nutrition, Immunity, and Infection in Infants and Children*. Vevey/Lippincott Williams & Wilkins, Philadelphia: 2001, pp 89-120.
- 6 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 Natl Acad Sci USA*. 2000;97:7078-7083.
- 7 Heird WC: in Nelson Behrman RE, Kliegman RM, Jenson FB (eds); *Food Insecurity, Hunger and Undernutrition*; 17th ed., Chapter 42, WB Saunders Company Philadelphia, 2004, pp 167-173.
- 8 Dai G, McMurray DN: Altered cytokine production and impaired antimycobacterial immunity in protein-malnourished guinea pigs. *Infect Immun* 1998;66:3562-3568.
- 9 Badr G, Sayed D, Alhazaa I, Elsayh K, Ahmed E, Alwasel S: T lymphocytes from malnourished infants are short-lived and dysfunctional cells. *Immunobiology* 2011;216:309-315.
- 10 Rodríguez L, González C, Flores L, Graniel J, Ortiz R: Assessment by flow cytometry of cytokine production in malnourished children. *Clin Diagn Lab Immunol* 2005;12:502-507.
- 11 González-Martínez H, Rodríguez L, Nájera O, Cruz D, Miliar A, Domínguez A, Sánchez F, Graniel J, González-Torres MC: Expression of cytokine mRNA in lymphocytes of malnourished children. *J Clin Immunol* 2008;28:593-599.
- 12 Fock RA, Vinolo MA, de Moura Sá Rocha V, de Sá Rocha LC, Borelli P: Protein-energy malnutrition decreases the expression of TLR-4/MD-2 and CD14 receptors in peritoneal macrophages and reduces the synthesis of TNF-alpha in response to lipopolysaccharide (LPS) in mice. *Cytokine* 2007;40:105-114.
- 13 Kisseleva T, Bhattacharya S, Braunstein J, Schindler W: Signaling through the JAK/STAT pathway, recent advances and future challenges. *Gene* 2002;285:1-24.

- 14 Hand T, Cui W, Jung Y, Sefik E, Joshi NS, Chande A, Liu Y, Kaech SM: Differential effects of STAT5 and PI3K/AKT signaling on effector and memory CD8 T-cell survival. *Proc Natl Acad Sci USA* 2010;107:16601-16606.
- 15 El-Hodhod MA, Nassar MF, Zaki MM, Moustafa A: Apoptotic changes in lymphocytes of protein energy malnutrition children. *Nutr Res* 2005;25:21-29.
- 16 Nassar M, El-Batrawy S, Nagy N: CD95 expression in white blood cells of malnourished infants during hospitalization and catch-up growth. *East Mediterr Health J* 2009;15:574-83.
- 17 Moser B, Loetscher P: Lymphocyte traffic control by chemokines. *Nat Immunol* 2001;2:123-128.
- 18 Thelen M: Dancing to the tune of chemokines. *Nat Immunol* 2001;2:129-134.
- 19 Badr G, Borhis G, Treton D, Moog C, Garraud O, Richard Y: HIV type 1 glycoprotein 120 inhibits human B cell chemotaxis to CXCL12, CC chemokine ligand (CCL)20, and CCL21. *J Immunol* 2005;175:302-310.
- 20 Badr G, Borhis G, Treton D, Richard Y: IFN α enhances human B-cell chemotaxis by modulating ligand-induced chemokine receptor signaling and internalization. *Int Immunol* 2005;17:459-467.
- 21 Badr G, Bédard N, Abdel Hakeem M, Trautmann L, Willems B, Villeneuve J, Haddad E, Sékaly R, Bruneau J, Shoukry N: Early interferon therapy for HCV rescues polyfunctional virus-specific CD8⁺ long-lived memory T cells. *J Virol* 2008;82:10017-10031.
- 22 Hasselbalch H, Ersboll AK, Jeppesen DL, Nielsen MB: Thymus size in infants from birth until 24 months of age evaluated by ultrasound. *Acta Radiol* 1999;40:41-44.
- 23 Collinson AC, Moore SE, Cole TJ, Prentice AM: Birth season and environmental influences on patterns of thymic growth in rural Gambian infants. *Acta Paediatr* 2003;92:1014-1020.
- 24 Savino W: The thymus gland is a target in malnutrition. *Eur J Clin Nutr* 2002;56:S46-49.
- 25 Nathan C, Sporn M: Cytokines in context. *J Cell Biol* 1991;113:981-986.
- 26 Malek TR: T helper cells, IL-2, and the generation of cytotoxic T-cell responses. *Trends Immunol* 2002;23:465-467.
- 27 Moon J, Nelson B: Phosphatidylinositol 3-kinase potentiates, but does not trigger, T cell proliferation mediated by the IL-2 receptor. *J Immunol* 2001;167:2714-2723.
- 28 Lali V, Crawley J, McCulloch D, Foxwell B: A late, prolonged activation of the phosphatidylinositol 3-kinase pathway is required for T cell proliferation. *J Immunol* 2004;172:3527-3534.
- 29 Mui A, Wakao L, Harada N, O'Farrell A, Miyajima A: Interleukin-3, granulocyte-macrophage colony-stimulating factor, and interleukin-5 transduce signals through two forms of STAT5. *J Leukoc Biol* 1995;57:799-803.
- 30 Uetrecht AC, Bear JE: Coronins: the return of the crown. *Trends Cell Biol* 2006;16:421-426.
- 31 Gomez T, Hamann M, McCarney S, Savoy D, Lubking C, Heldebrandt M, Labno C, McKean D, McNiven M, Burkhardt J, Billadeau D: Dynamin 2 regulates T cell activation by controlling actin polymerization at the immunological synapse. *Nat Immunol* 2005;6:261-270.