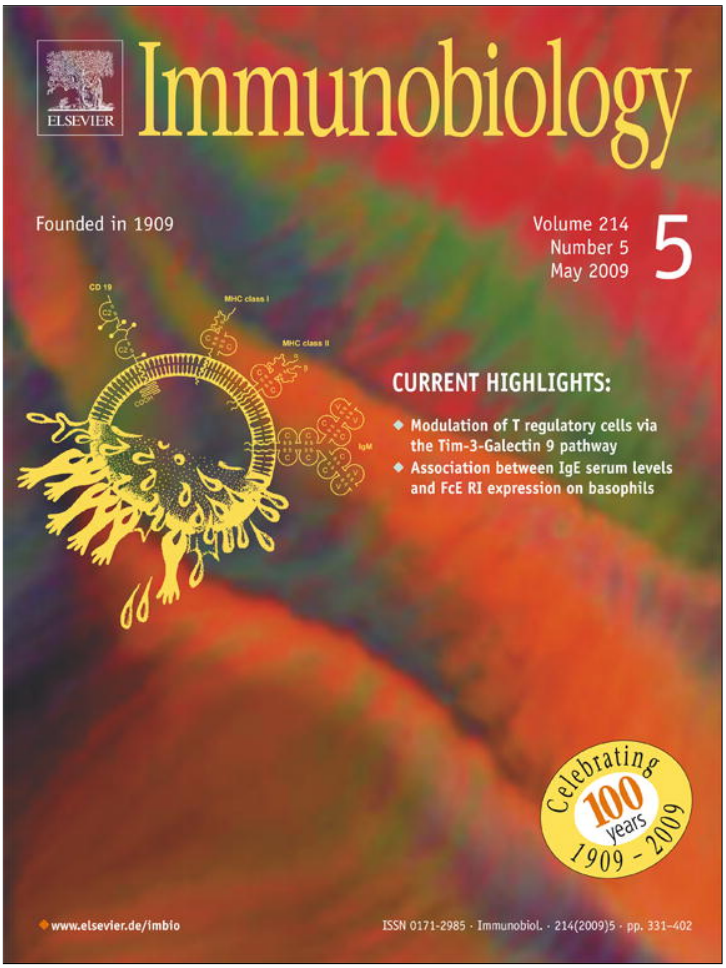

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Flow cytometric analysis of T-lymphocytes from nasopharynx-associated lymphoid tissue (NALT) in a model of secondary immunodeficiency in Wistar rats

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Abstract

Nasopharynx-associated lymphoid tissue (NALT) is responsible for immune responses in the upper respiratory tract of rodents. In our model of protein malnutrition (R21 group), bronchus-associated lymphoid tissue (BALT), situated in the lower respiratory tract, showed a decrease of $CD4^+$, $CD8\alpha^+$, and $TCR\alpha\beta^+$ lymphocytes but $TCR\gamma\delta^+$ cells were increased. Besides, there is no information regarding the frequencies of T-cell populations in 60-day-old Wistar rats (C60 group). So, the aim of the present study was to analyze by flow cytometry NALT T-cells from both groups. NALT lymphocytes were isolated from R21 and C60 groups and stained with different antibodies. Samples were run on a FACScalibur flow cytometer. Background staining was evaluated using isotype controls. Data analysis was performed using BD Cell Quest® and WinMDI 2.9. In C60, the predominant population was $CD4^+TCR\alpha\beta^+$, which was significantly diminished in the R21 group. However, $CD8\alpha^+$, the majority expressing $CD8\alpha\beta$, and $TCR\gamma\delta^+$ cells were not affected. In our model of secondary immunodeficiency, there is a compartmentalization between NALT and BALT because they differ in the populations affected even though they are inductive sites of the respiratory tract in the common mucosal immune system.

Keywords: CD4; Malnutrition; NALT; Secondary immunodeficiency; $TCR\alpha\beta$; $TCR\gamma\delta$; Wistar rats

Introduction

Mucosa-associated lymphoid tissue (MALT) provides a morphological and functional distinct line of protection characteristic of mucosal surfaces, consisting of lymphocytes and lymphoid aggregates in the lamina propria and in the submucosa of the mucosal membranes. MALT is represented in the lower respiratory tract of rodents by bronchus-associated lymphoid tissue (BALT), while in the upper respiratory tract, the organ

Abbreviations: BALT, bronchus-associated lymphoid tissue; FITC, fluorescein isothiocyanate; GALT, gut-associated lymphoid tissue; HBSS–FBS, Hank's balanced salt solution-fetal bovine serum; MALT, mucosa-associated lymphoid tissue; NALT, nasopharynx-associated lymphoid tissue; PBS, phosphate-buffered saline; PE, R-phycoerythrin; SAv, streptavidin; TNP, trinitrophenol.

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involved in immune responses is a lymphoid structure called nasopharynx-associated lymphoid tissue (NALT). NALT is a paired and rod-shaped organ situated in the floor of the nasal cavity at the entrance of the bifurcated nasopharyngeal duct and it is found earlier in ontogeny than BALT, perhaps due to its strategic position as the mucosa that lines the nasal cavity is the first site of contact with the inhaled air laden with antigens (Kraal, 2005).

Previous studies from our laboratory, performed in an experimental model of immunodeficiency in which rats are fed a protein-free diet from weaning until they lose 25% of their initial body weight, and then fed with a 20% casein diet during 21 days, have shown permanent severe alterations in the thymus, gut and gut-associated lymphoid tissue (GALT) (López, 1987; López et al., 1989; Melton and Roux, 1989; Slobodianik et al., 1989). Furthermore, similar findings were observed in BALT (Márquez, 1999).

In an ontogenic study of NALT, we have demonstrated that even though the TCR$\gamma\delta^+$ population predominates at 7 days of age, as in BALT, in 60-day-old control animals there was a predominance of CD4$^+$ and TCR$\alpha\beta^+$ T-cells (Sosa and Roux, 2004). Besides, the majority of CD8α^+ lymphocytes expressed the heterodimeric isoform, i.e., CD8$\alpha\beta$, thus differing from BALT (Sosa et al., 2004). Moreover, in our experimental model results obtained in BALT were different when compared with the age-matched control group fed with stock diet (Sosa et al., 2004).

Therefore, the aim of the present study was to compare by flow cytometry the T-cell populations on isolated NALT lymphocytes from control and refed rats.

Materials and methods

Animals and diets

The project has been approved by the Ethical Committee of the School of Pharmacy and Biochemistry, University of Buenos Aires, Argentina. Weanling Wistar rats (closed colony from the breeding unit kept at the animal facilities of the School of Pharmacy and Biochemistry) of either sex, aged 21–23 days, were weighed and randomly divided into two groups.

Rats from one group ($N = 28$) were fed a protein-free diet until they lost 25% of their initial body weight (34–41 days of age) and then fed with a diet containing 20% casein, as the only source of protein, for 21 days (R21 group, 55–62 days of age).

An age-matched and well-nourished control group, receiving stock diet (Cooperación, Argentina, 23% protein) from weaning ($N = 25$), was run simultaneously until they were 60 days old (C60 group).

Experimental isocaloric diets (4.05 kcal/g) were prepared as previously reported (Slobodianik et al., 1989). To a basal concentrated diet containing all the essential nutrients, as recommended by the American Institute of Nutrition (1977), casein was omitted and replaced by dextrin in the protein-free diet. However, in the repletion diet, which is nutritionally adequate, casein was incorporated to provide 20% protein and then filled up to 100 g by adding dextrin.

Water and diets were offered *ad libitum*. During all the experimental time, rats were exposed to 12 h of light and 12 h of darkness and room temperature was kept at $21 \pm 1\,^{\circ}C$.

Isolation of NALT

NALT was isolated as previously described (Sosa and Roux, 2004). Briefly, rats were killed by decapitation under anesthesia with diethyl ether. The lower jaws and the tongues were removed and the palates were excised with a scalpel blade from behind the incisor teeth to the molar teeth. Then, palates were gently pulled from front to rear with fine forceps, carefully dissecting them from the underlying bone tissue with the scalpel blade.

Preparation of cell suspensions

Palates, containing NALT, were immediately placed in a Petri dish containing ice-cold Hank's balanced salt solution supplemented with 5% heat-inactivated fetal bovine serum (HBSS–FBS) and teased gently against a stainless steel mesh immersed in the medium in order to release cells. NALT cell suspensions from four or five animals were pooled and washed three times with HBSS–FBS by centrifugation. Then, erythrocytes were eliminated by the addition of 0.83% NH_4Cl solution. The dissociated cell suspensions were filtered through nylon wool to remove large cellular aggregates and detritus and then washed three times with HBSS–FBS by centrifugation. Viability of cell preparations routinely exceeded 90% as judged by Trypan Blue staining.

Antibodies

Staining of T-cells was performed using the following monoclonal antibodies (BD PharMingen, San Diego, CA, USA) at the appropriate concentrations as determined by titration: fluorescein isothiocyanate (FITC)-conjugated mouse $IgG_{1,\kappa}$ anti-rat CD5 (clone OX-19) followed by FITC-conjugated goat $F(ab')_2$ fragment to mouse IgG (whole molecule) (Cappel ICN, Aurora, OH, USA); FITC-conjugated mouse $IgG_{1,\kappa}$ anti-rat CD8α (clone OX-8); biotin-conjugated mouse $IgG_{1,\kappa}$ anti-rat

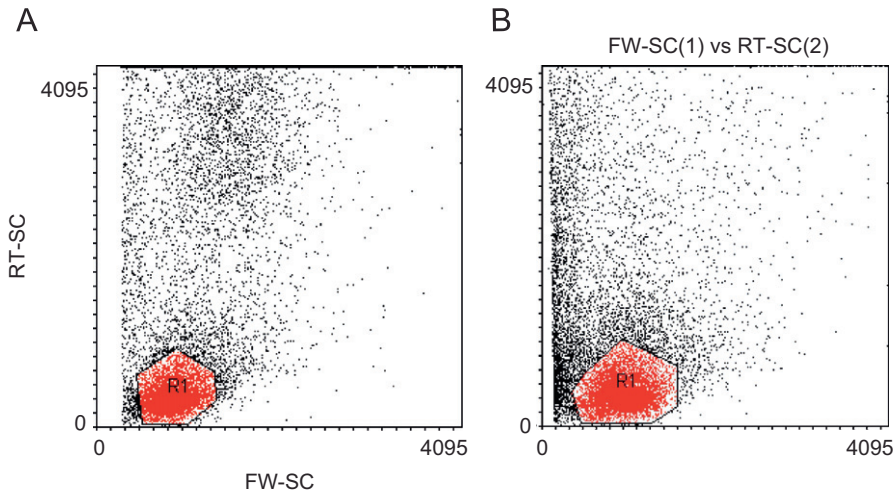

Fig. 1. Flow cytometric analysis of NALT lymphocytes. Forward (FW-SC) and side scatter (RT-SC) of NALT lymphocytes isolated from control Wistar rats of 60 days of age (A) and from R21 animals (B). The gated populations (R1) represent NALT lymphoid cells.

CD8β (clone 341), followed by streptavidin Cy-Chrome® conjugate (SAv-Cy-Chrome®); R-Phycoerythrin (PE)-conjugated mouse $IgG_{2a,\kappa}$ anti-rat CD4 (clone OX-38); PE-conjugated mouse $IgG_{1,\kappa}$ anti-rat T-cell receptor (TCR) $\alpha\beta$ (clone R73); FITC- or PE-conjugated mouse $IgG_{1,\kappa}$ anti-rat TCR$\gamma\delta$ (clone V65); and purified mouse $IgG_{2a,\kappa}$ anti-rat CD4 (clone OX-38) followed by FITC-conjugated goat $F(ab')_2$ fragment to mouse IgG (whole molecule) purchased from Cappel (ICN, Aurora, OH, USA).

Background staining was evaluated using the following isotype controls: FITC-conjugated mouse $IgG_{1,\kappa}$ monoclonal immunoglobulin isotype standard (anti-trinitrophenol –TNP–); Cy-Chrome®-conjugated mouse $IgG_{1,\kappa}$ monoclonal immunoglobulin isotype control; PE-conjugated mouse $IgG_{2a,\kappa}$ monoclonal immunoglobulin isotype standard (anti-TNP); PE-conjugated mouse $IgG_{1,\kappa}$ monoclonal immunoglobulin isotype standard (anti-TNP); mouse $IgG_{1,\kappa}$ monoclonal immunoglobulin isotype standard (anti-TNP); and mouse $IgG_{2a,\kappa}$ monoclonal immunoglobulin isotype standard (anti-TNP) followed by FITC-conjugated goat $F(ab')_2$ fragment to mouse IgG (whole molecule).

Flow cytometric analysis

Pooled cells obtained from four or five animals, either of the control or of the experimental group, were phenotypically characterized using a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Data were analyzed for at least 10,000 events with BD Cell Quest® version 3.3 (Becton Dickinson, San José, CA, USA) and WinMDI version 2.9 (The Scripps Research Institute, San Diego, CA, USA). Isotype-matched antibodies were used as negative controls, and for cell analysis the gate was initially set on the lymphoid fraction selected by the forward angle (FSC) and 90° light scatter (SSC) from NALT lymphocytes (Fig. 1). After the enrichment of NALT cells with nylon wool, cell staining was performed using mouse anti-rat CD5 antibody, which is expressed on all T-lymphocytes, in order to control the enrichment procedure. More than 90% of the cells in the gated region were T-lymphocytes. Results are expressed as the percentage of positively stained cells in the total cell population exceeding the background-staining signal in at least three independent experiments.

NALT cells were stained with the appropriate monoclonal antibodies, directly or indirectly, suspended in 2% paraformaldehyde in phosphate-buffered saline (PBS), and stored in the dark at 4 °C until cytometric analysis. Briefly, 5×10^5 NALT cells were incubated for 30 min on ice with these antibodies alone or mixed in the following combinations: (a) FITC-conjugated anti-CD8α and biotin-conjugated anti-CD8β followed by streptavidin-Cy-Chrome; (b) FITC-conjugated anti-CD8α and PE-conjugated anti-TCR$\gamma\delta$; (c) FITC-conjugated anti-CD8α and PE-conjugated anti-TCR$\alpha\beta$; (d) anti-CD4 followed by FITC-conjugated goat $F(ab')_2$ to mouse IgG (whole molecule), and PE-conjugated mouse anti-rat TCR$\alpha\beta$; and (e) PE-conjugated anti-CD4 and FITC-conjugated anti-TCR$\gamma\delta$.

Statistical evaluation

The analysis for each subset was performed independently at least three times and results are expressed as mean ± SEM. Normality was tested using Shapiro-Wilks' test, modified by Mahibbur and Govindarajulu, and the Q–Q plot. Statistical analyses were performed

using Student's *t* test with InfoStat version 2008, Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina.

Table 1. Percentages of NALT T-cell populations from control and protein-refed Wistar rats[a].

Phenotype	C60	R21
$CD4^+$	61.2±3.0	23.8±0.6*
$CD8\alpha^+$	26.3±0.2	25.8±0.1
$CD8\beta^+$	20.1±1.3	19.1±1.0
$TCR\alpha\beta^+$	55.8±7.9	33.4±4.5**
$TCR\gamma\delta^+$	2.5±0.3	2.6±0.3

[a]Cells were isolated as described in Materials and Methods. Results are expressed as mean±SEM of the percentage of positive cells in at least three experiments with pools obtained from five rats. C60: control group, R21: protein-refed Wistar rats. Analyses were performed using two-tailed Student's *t* test.

*$p = 0.0002$.

**$p = 0.0279$.

Results

Results obtained from flow cytometric analyses performed on the different rat NALT T-cell subpopulations from experimental (R21) and age-matched control (C60) Wistar rats are summarized in Table 1.

These results showed differences between both groups only in $CD4^+$ and in $TCR\alpha\beta^+$ T-cells and representative histograms for both subsets are shown in Fig. 2. The majority of T-cells in NALT from control rats belonged to the $CD4^+$ and $TCR\alpha\beta^+$ populations, with approximately 60% and 56% of the total number of lymphocytes, respectively, and they were significantly affected in the experimental group (R21) (Table 1). There were no significant differences in the rest of the subsets, and the representative histograms are shown in Fig. 3.

The flow cytometric analyses in control Wistar rats also indicated that the majority of T-lymphocytes co-expressed $TCR\alpha\beta$ and CD4 (Table 2). This population is significantly diminished in the experimental group when compared with the control group (Table 2 and Fig. 4).

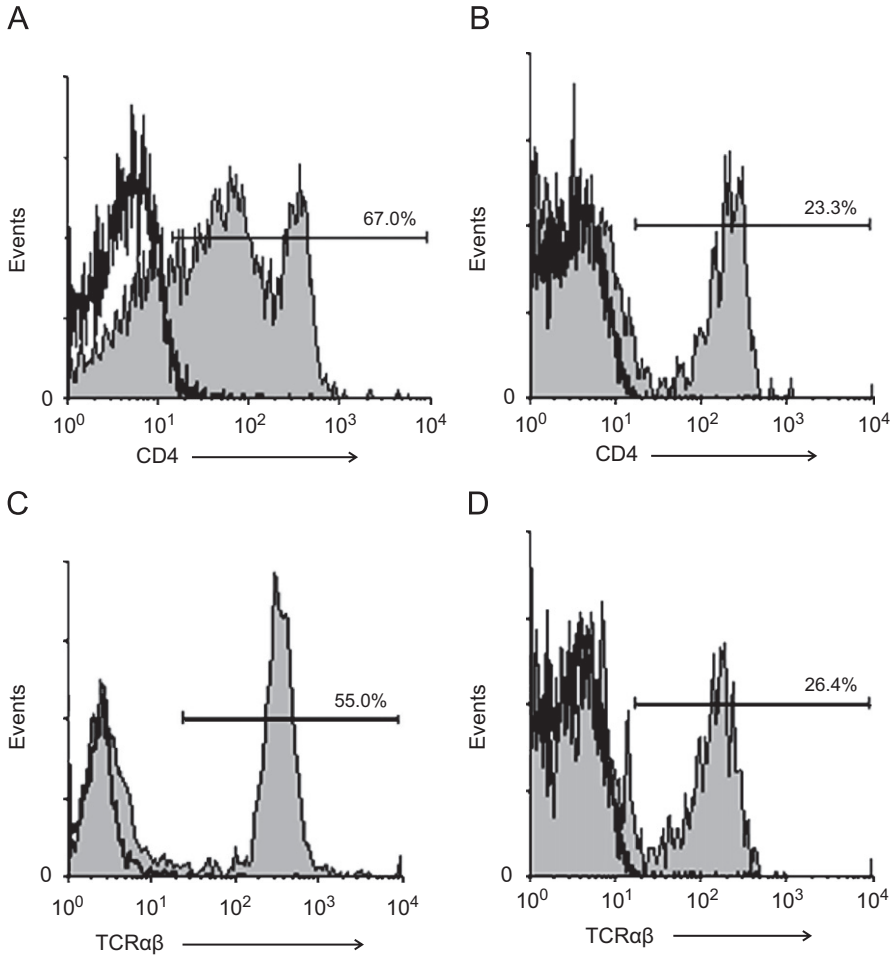

Fig. 2. Histograms showing $CD4^+$ and $TCR\alpha\beta^+$ T-cells isolated from control Wistar rats of 60 days of age (A and C), and from protein refed rats (R21 group) in B and D. Results are representative of at least three independent experiments. The percentage of positive cells is indicated in each histogram.

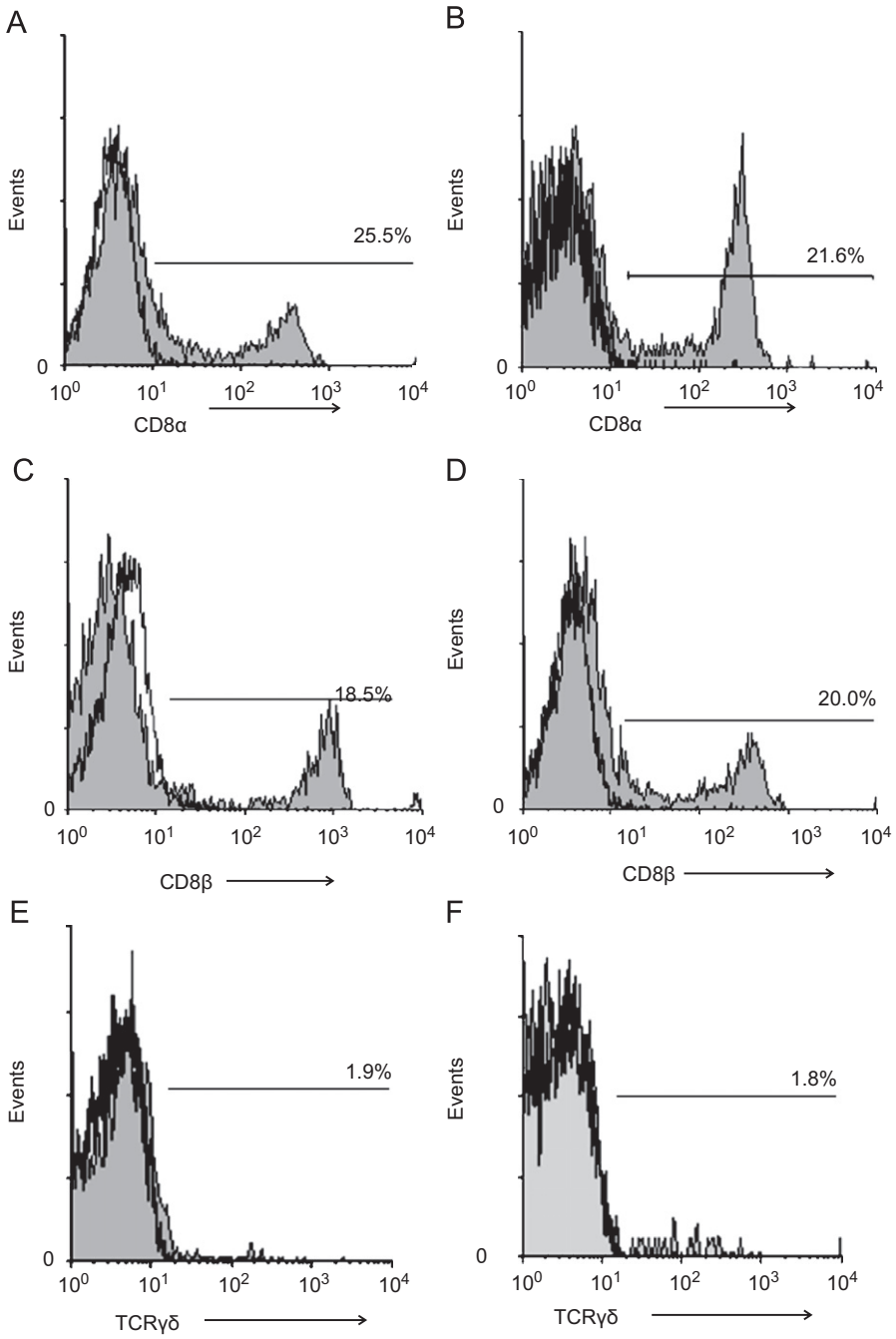

Fig. 3. Histograms showing CD8α^+ (A and B), CD8β^+ (C and D), and TCR$\gamma\delta^+$ (E and F) T-lymphocytes isolated from control Wistar rats of 60 days of age (A, C, and E) and from protein refed rats (R21 group) in B, D, and F. Results are representative of at least three independent experiments. The percentage of positive cells is indicated in each histogram.

Our results also demonstrate that in both groups (C60 and R21) the majority of the CD8α^+ population expressed the heterodimeric form, i.e., CD8$\alpha\beta$, and that no differences could be observed in CD8$\alpha\alpha$ (CD8α^+CD8β^-) and CD8$\alpha\beta$ (CD8α^+CD8β^+) subpopulations (Table 1). And with respect to the TCR$\gamma\delta$ population, its frequency was very low and no differences could be observed between both groups (Table 1). In addition, when studying the co-expression of CD4 and TCR$\alpha\beta$, CD4 and TCR$\gamma\delta$, and of CD8α and TCR$\gamma\delta$, no significant differences were observed (Table 2 and Fig. 5).

Discussion

The NALT is a paired rod-shaped organ situated in the floor of the nasal cavity at the entrance of the

nasopharyngeal duct and is responsible for the immune responses in the upper respiratory tract. This is the first report on the complete characterization of the different T-cell populations by flow cytometry not only in 60-day-old control Wistar rats but also in our model of secondary immunodeficiency.

In a previous published study on the ontogeny of T-lymphocytes in the NALT from growing Wistar rats, we determined the predominance of $CD4^+$ and $TCR\alpha\beta^+$ subsets at 60 days of age (Sosa and Roux, 2004). Studies performed by other authors in rats have demonstrated only a predominance of $TCR\alpha\beta^+$ cells as no other T-cell populations were studied by flow cytometry in cell suspensions (Koornstra et al., 1993). Besides, in C57BL/6 mice between 6 and 8 weeks of age, a higher frequency of $CD4^+$ and $TCR\alpha\beta^+$ T-cells is observed (Hiroi et al., 1998). The predominance of $CD4^+$ T-lymphocytes and the CD4/CD8 ratio of 2.3 are characteristic of mucosal inductive sites, and this is generally consistent with the two aforementioned studies (Hiroi et al., 1998; Koornstra et al., 1993).

Table 2. Co-expressions of the different phenotypes in NALT from control and protein-refed Wistar rats[a].

Phenotypes	C60	R21
$CD4^+CD8\alpha^-TCR\alpha\beta^+$	43.4±2.0	22.7±1.3*
$CD4^+CD8\alpha^-TCR\gamma\delta^+$	0.7±0.2	1.0±0.4
$CD8\alpha^+CD4^-TCR\alpha\beta^+$	5.0±0.3	5.1±0.7
$CD8\alpha^+CD4^-TCR\gamma\delta^+$	1.9±0.3	1.4±0.1

[a]Cells were isolated as described in Materials and Methods. Results are expressed as mean±SEM of the percentage of positive cells in at least three experiments with pools obtained from five rats. C60: control group, R21: protein-refed Wistar rats. Analyses were performed using two-tailed Student's t test.

*$p = 0.0010$.

In the present paper, in addition to the predominance of $CD4^+$ and $TCR\alpha\beta^+$ subsets in 60-day-old rats, we observed that the majority of the $CD8\alpha^+$ population expresses the $CD8\alpha\beta$ isoform and that the $TCR\gamma\delta$ subset was very low compared with the $TCR\alpha\beta$. Besides, when comparing the T-cell subsets from 60-day-old rats versus the experimental ones, there was a greatly significant decrease of $CD4^+$ and $TCR\alpha\beta^+$ subpopulations. The $CD8\alpha^+$, $CD8\beta^+$ and $TCR\gamma\delta^+$ subsets showed no significant differences.

The study of the co-expression of CD4 and $TCR\alpha\beta$, CD4 and $TCR\gamma\delta$, $CD8\alpha$ and $TCR\alpha\beta$, and of $CD8\alpha$ and $TCR\gamma\delta$ showed only a significant decrease in the $CD4^+TCR\alpha\beta^+$ population in the experimental group versus the control group.

It is well known that all forms of immunity are affected by protein-energy malnutrition in young children. It is especially important the synergy between malnutrition and infection, in terms of its consequences on children mortality. These consequences are even more evident in children suffering from severe protein-energy malnutrition. Our experimental model is similar to kwashiorkor, one of the forms of this type of malnutrition in humans in which there is a predominance of protein deficiency. But there are no studies relative to the impact of the severe protein deficiency in the organs of the common mucosal immune system of young children. The only studies available were performed in peripheral blood either to study the effects on antibody formation or on different T-cell subsets. Two studies performed in the 1960s demonstrated that the failure of antibody formation is reversed within a few days of protein therapy (Brown and Katz, 1966; Fernández, 1960). Besides, when considering T-cell subpopulations, Chandra et al. have shown a decrease in CD4+ and CD8+ T-cells, and in the ratio

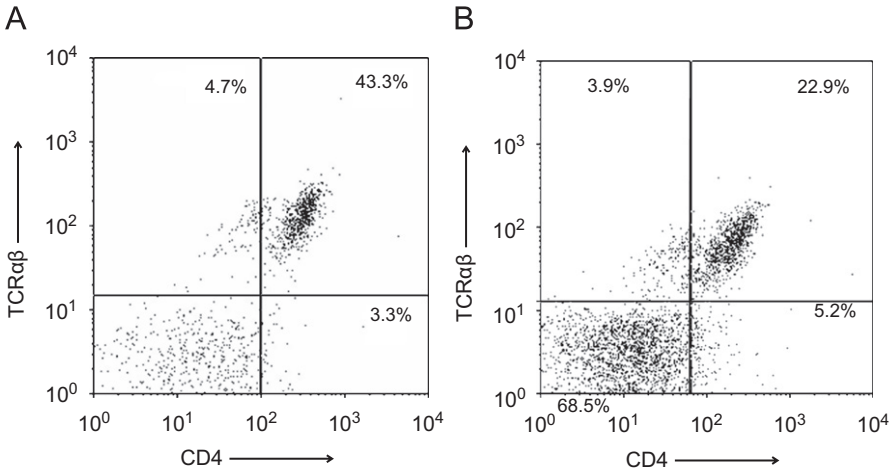

Fig. 4. Dot plot graphs of $CD4^+TCR\alpha\beta^+$ T-cells obtained from control (A) and experimental (B) rats as described in Materials and Methods. Results are representative of at least three independent experiments. The percentage of cells localized in each quadrant is indicated.

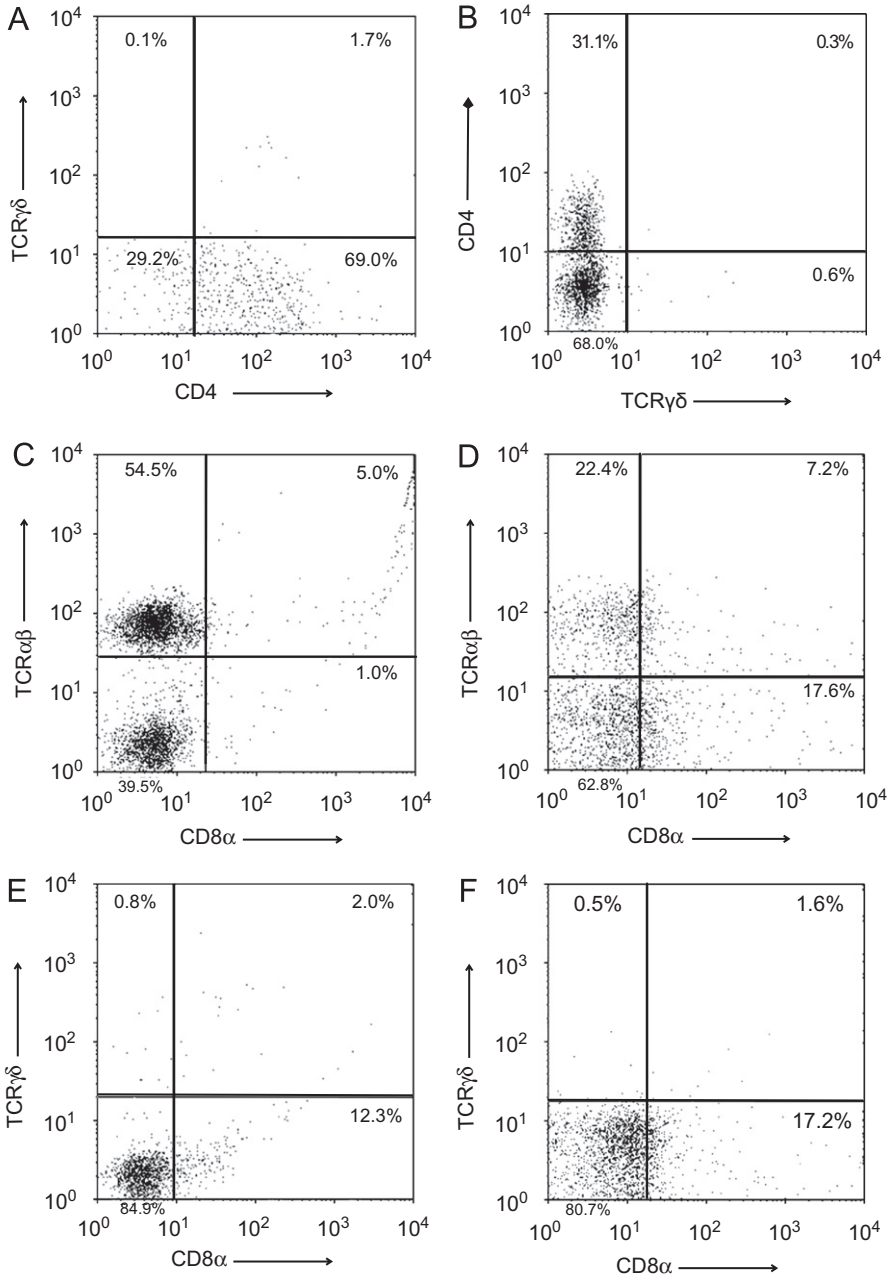

Fig. 5. Dot plot graphs of $CD4^+TCR\gamma\delta^+$, $CD8\alpha^+TCR\alpha\beta^+$, and $CD8\alpha^+TCR\gamma\delta^+$ T-cells obtained from control (A, C, and E) and from experimental (B, D, and F) rats as described in Materials and Methods. Results are representative of at least three independent experiments. The percentage of cells localized in each quadrant is indicated.

CD4+: CD8+. They have also demonstrated that these alterations were reversed to normal following nutritional therapy for 4–8 weeks (Chandra et al., 1982).

In a paper published by Nájera et al., the effects of infection and malnutrition on the proportion of peripheral-lymphocyte subsets in well-nourished non-bacterium-infected, well-nourished bacterium-infected, and malnourished bacterium-infected children were assessed by flow cytometry. There were no significant differences in T-lymphocytes subsets between the last two groups of children regardless of the infection type, respiratory or gastrointestinal (Nájera et al., 2004). There are also two reports in peripheral blood in which no differences between the percentages of B lymphocytes in malnourished versus well-nourished children have been reported (Rafii et al., 1977; Stiehm, 1980).

No studies have been performed in the immune organs of the oronasopharyngeal region in humans. In the rat, the mucosal immune system of the respiratory tract is represented by NALT, in the upper respiratory tract, and BALT, in the lower respiratory tract. In our studies in a rat model of secondary immunodeficiency,

there are differences between NALT and BALT. In NALT, there is only a decrease of $CD4^+$ and $TCR\alpha\beta^+$ populations whereas in BALT there is a decrease in $CD4^+$, $TCR\alpha\beta^+$, and $CD8\alpha^+$ T populations. Another difference is that in BALT the number of $TCR\gamma\delta^+$ lymphocytes is increased in the experimental group (Sosa et al., 2004). Moreover, only in BALT, the decrease in the number of $CD4^+$ cells was accompanied by a decrease in IgA^+ B cells (Márquez, 1999; Sosa et al., 2004). Therefore, even though NALT and BALT are inductive sites of the respiratory tract there is a compartmentalization between them with respect to the deleterious effects of severe protein deficiency at weaning, even after the recovery period with a 20% casein diet during 21 days. The effects of secondary immunodeficiency on certain T-cell populations could not be reversed by the administration of casein, which is the protein with the highest biologic value (80–85).

In conclusion, the data presented in this study reveal profound qualitative differences at the mucosal sites from malnourished rats, suggesting that, during development, immunological alterations due to nutritional status may be irreversible, probably affecting the role of mucosal immune system in the host defense against inhalatory agents. In this regard, the present study differs from what has been published in peripheral blood from malnourished and infected children. Unfortunately, our results in the mucosal immune system from malnourished rats cannot be compared with similar studies in humans.

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