Short Communication

Dietary zinc deficiency lowers the proportions of splenic CD90+ (Thy-1+) B-cells and late thymic emigrant T-cells in growing rats

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Zn-deficient (ZD) rats have a lower proportion of splenic CD90+T-cells which could be due to fewer new T-cells exiting the thymus, defective post-thymic maturation or increased cell death. Post-thymic maturation of splenic lymphocytes and their viability were determined by flow cytometry in weanling rats assigned to ZD (<1 mg Zn/kg; *ad libitum*), diet-restricted (DR; 30 mg Zn/kg; limited to the amount of feed as consumed by ZD rats), marginally Zn-deficient (MZD; 10 mg Zn/kg; *ad libitum*) or control (30 mg Zn/kg; *ad libitum*) groups for 3 weeks. ZD rats had a 29 % lower percentage of splenic CD90+T-cells and both ZD and DR rats had a 30 % lower proportion of splenic CD90+B-cells compared with control rats. When the splenic CD90+T-cells were characterised further, there was no difference among the groups in the first two stages of post-thymic development; however, ZD, DR and MZD rats had a 42 % lower proportion of late thymic emigrants (TCRαβ+CD90+CD45RC+RT6.1+) compared with control rats. There was no difference among groups in the proportion of splenic CD90+T-cells in the non-viable region; however, ZD rats had a higher proportion of CD90+B-cells in the non-viable region compared with MZD and control animals, suggesting that this phenotype was more susceptible to cell death during deficiency. The lower proportion of splenic CD90+T-cells in ZD rats does not appear to be due to a defect in thymic production or increased cell death in the spleen. Future studies should determine if late thymic emigrants have homed to other peripheral organs.

Zinc deficiency: CD90 T-cells: CD45RC T-cells: Rats

Zn-deficient (ZD) rats have a lower proportion of new splenic T-cells (CD90+) compared with diet-restricted (DR) and control rats[1]. CD90 (Thy-1) is found on newly released peripheral T-cells for approximately 3 d in rats[2]. Initially, both RT6 and CD45RC are expressed as CD90 disappears (3–11 d post-thymus) and 76 d after T-cells have been released from the rat thymus they express either CD45RC or RT6[3]. Further characterisation of the maturation of CD90+T-cells into CD90− T-cells using markers such as RT6.1 and CD45RC has not been investigated in a model of dietary Zn deficiency. We hypothesised that Zn deficiency alters the proportion of splenic T-cell phenotypes, resulting in fewer new cells in the spleen and thereby limiting the lymphocyte repertoire. Furthermore, recent thymic emigrant (CD90+RT6−) T-cells may be more susceptible to cell death[4], and this could contribute to the lymphopenia of Zn deficiency.

Thus, the objective of the present study was to determine the proportion of lymphocytes that express different combinations of TCRαβ, CD90, RT6.1 and CD45RC to further characterise the stage of maturity of splenic T- and B-cells in both marginal and severe Zn deficiency using the growing rat as a model. A secondary objective was to determine whether differences in the proportion of splenic lymphocyte subsets could be explained by altered cell viability.

Experimental methods

Animals and diets

Sprague–Dawley rats (age 3 weeks; Charles River Laboratories, St Constant, PQ, Canada) were randomly assigned to one of four dietary groups for 3 weeks: ZD (<1 mg Zn/kg); marginally Zn-deficient (MZD; 10 mg Zn/kg); nutritionally complete control diet (30 mg Zn/kg); DR (fed the control diet, limited to intake of paired ZD rat).

Sample collection

Rats were euthanised by CO_2 asphyxiation. Spleens were removed aseptically, weighed and processed immediately. Femurs were removed, stored at $-20°C$ and analysed for Zn by atomic absorption spectrometry[1].

Determination of splenic T-lymphocyte subpopulations

Antibodies. Anti-rat monoclonal antibodies against TCRαβ (PE label, clone R73, isotype mouse $IgG_{1,k}$), CD90 (PerCP label, Thy1.1, clone OX-7, isotype mouse $IgG_{1,k}$), RT6.1 (purified, clone P4/16, isotype rat $IgG_{2b,k}$), $IgG_{2b,k}$ (biotin label, RG7/11.1 clone, isotype mouse $IgG_{2b,k}$) and CD45RC (FITC label, clone OX-22, isotype mouse $IgG_{1,k}$) were obtained from BD Pharmingen (Mississauga, ON, Canada). The RT6.1 antibody was biotylated with the $IgG_{2b,k}$ and fluorescently labelled with streptavidin-PE-Cy7 conjugate.

Cell labelling and flow cytometry. Single cell suspensions were prepared by pressing tissues through nylon screens. Erythrocytes were lysed using ammonium chloride lysis buffer. Cells were incubated with antibodies for 30 min at 4°C, followed by a wash step. Cells were re-suspended in 1 % paraformaldehyde and stored overnight at 4°C before analysis on a Beckman Coulter EPICS ALTRA (Beckman Coulter, Mississauga, ON, Canada) high-speed cell sorter with laser excitation tuned to 488 nm (65 mW). Forward versus side scatter histograms were used to identify intact lymphoid cells, non-viable cells, or granulocytes.

Statistical analyses

Data were analysed by one-way ANOVA and Duncan's new multiple-range test was used for means testing (SAS 8.2; SAS Institute Inc., Cary, NC, USA). Significance was set at $P < 0.05$.

Results

Body weight, spleen weight and zinc status

ZD rats (142 (SEM 4) g) had a 49 % lower body weight compared with MZD (274 (SEM 5) g) and control (280 (SEM 5) g) rats. Despite consuming the same amount of feed, ZD rats weighed 15 % less than DR (168 (SEM 3) g) animals. Spleen weight was not different among groups when corrected for body weight (data not shown). Femur Zn concentrations were 65 % lower in ZD (1.09 (SEM 0.05) μmol/g) compared with MZD (3.13 (SEM 0.05) μmol/g) rats, 25 % lower in MZD compared with DR (4.14 (SEM 0.12) μmol/g) rats, and 15 % lower in DR compared with control (4.85 (SEM 0.16) μmol/g) rats.

Size and granularity of splenocytes

ZD rats had 242 % more cells in the granulocyte gate than DR, MZD and control rats (4.1 (SEM 0.7), 1.2 (SEM 0.2), 1.2 (SEM 0.2) and 1.3 (SEM 0.2) %, respectively). There was no difference in the proportion of splenocytes that appeared in the viable or non-viable lymphocyte gates among treatment groups (data not shown).

Proportions of splenic CD90+ T- and B- lymphocytes

ZD rats had a 29 % lower proportion of new splenic T-cells ($TCRαβ^+CD90^+$) in the viable region compared with control rats, but the ZD group was not significantly different from the DR and MZD groups (Fig. 1 (a)). Both ZD and DR rats had approximately a 30 % lower proportion of new B-cells ($TCRαβ^-CD45RC^+CD90^+$) in the viable region compared with MZD and control rats.

There was no difference among groups in the proportion of new splenic T-cells in the non-viable region (Fig. 1 (b)). ZD rats had a 61–73 % higher proportion of new B-cells in the non-viable region compared with control and MZD rats, but were not different from DR rats.

Stages of splenic T- lymphocyte maturity

There were no differences among groups in the proportion of the most recent thymic emigrants or the first stage of late thymic emigrants (Table 1). However, ZD, DR and MZD rats had 42 % fewer late thymic emigrants ($TCRαβ^+CD90^+CD45RC^{+-}RT6.1^+$) compared with control animals. There was no difference in the proportion of late thymic emigrants in the non-viable region (data not shown). There were no differences among ZD, DR and control rats in the proportion of early mature peripheral T-cells ($TCRαβ^+CD90^-CD45RC^+RT6.1^+$), but MZD rats had 40 % fewer of these cells compared with all the other dietary treatment groups. Once again, there was no difference in the proportion of early mature peripheral T-cells in the
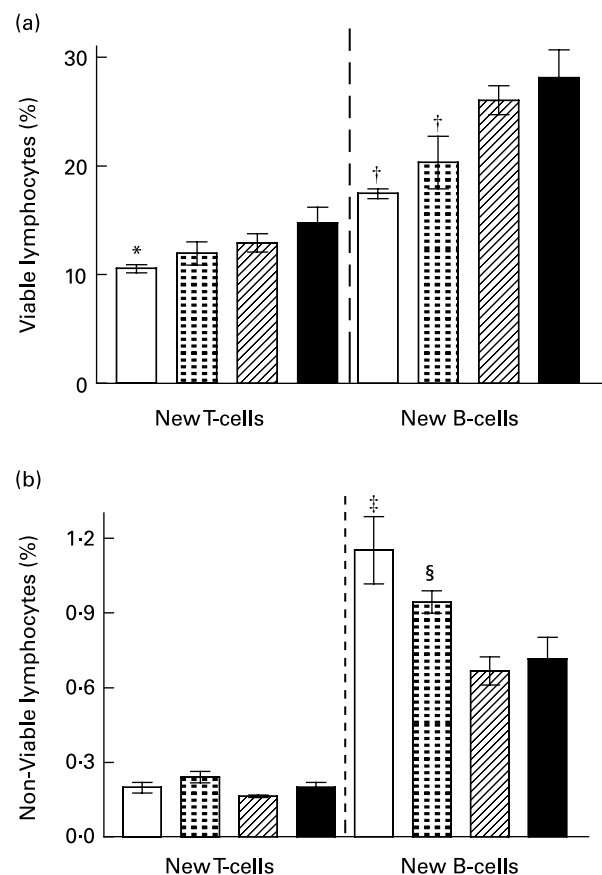

Fig. 1. Proportion of splenic new T-cells ($TCRαβ^+CD90^+$) and new B-cells ($TCRαβ^-CD45RC^+CD90^+$) in the viable (a) and non-viable (b) gates by flow cytometry. Values are means for six rats, with standard errors represented by vertical bars. (□), Zn-deficient group; (▦), diet-restricted group; (▨), marginally Zn-deficient group; (■), control group. *Mean value for new T-cells was significantly different from that of the control group ($P = 0.0488$). † Mean value for new B-cells was significantly different from that of the marginally Zn-deficient and control groups ($P = 0.0023$). ‡ Mean value for new B-cells was significantly different from that of the marginally Zn-deficient and control groups ($P = 0.0031$). § Mean value for new B-cells was significantly different from that of the marginally Zn-deficient group ($P = 0.0031$).

Table 1. Flow cytometric analysis of splenic T-lymphocyte subpopulations of zinc-deficient (ZD), marginally zinc-deficient (MZD), diet-restricted (DR) and control rats based on the Kaminga et al.[3] theory of post-thymic T-lymphocyte development in the rat‡

(Mean values with their standard errors)

	Thymus							Periphery						
		New peripheral T-cells						Mature peripheral T-cells						
		Recent thymic emigrants		Late thymic emigrants				Early		Intermediate		Late			
Subpopulation ...	CD90+ CD45RC- RT6.1-	CD90+ CD45RC- RT6.1-		CD90+ CD45RC- RT6.1+		CD90+ CD45RC+ RT6.1+		CD90- CD45RC+ RT6.1+		CD90- CD45RC- RT6.1-		CD90- CD45RC- RT6.1+		CD90- CD45RC+ RT6.1-	
		0–1 d		1–3 d				3–11 d		11–76 d		76+ d			
Group		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
ZD		7·3	0·7	3·0	0·4	3·9*	0·7	19·3	1·4	24·2	2·6	38·0	3·1	3·4	0·7
DR		8·6	0·7	3·3	0·5	3·8*	0·3	17·8	1·9	18·3§	2·7	44·1§	1·6	3·3	0·6
MZD		8·6	0·6	3·6	0·4	3·4*	0·4	11·3†	1·0	22·9	2·4	45·1	2·9	3·5	0·7
Control		6·4	1·0	3·7	0·4	6·4§	0·8	19·8	1·9	17·0	2·4	38·9	2·9	4·2	0·6

* Mean value within a column was significantly different from that of the control group ($P = 0.0084$).
† Mean value within a column was significantly different from that of the ZD, DR and CON groups ($P = 0.0043$).
‡ Values are percentages of $TCR\alpha\beta^+$ lymphocytes for six rats per group.
§ Five rats per group due to removal of outliers greater than 3 SD from the mean.

non-viable region (data not shown). There were no differences among the dietary treatment groups at the intermediate and end stages of T-cell maturation.

Discussion

The results confirm our previous report that ZD rats have a smaller proportion of new splenic T-cells ($CD90^+TCR\alpha\beta^+$) compared with control[1], and show that the lower proportion of new T-cells (Fig. 1 (a)) is specifically due to a lower proportion of the late thymic emigrants (Table 1).

One possibility for the lower proportion of late thymic emigrants in ZD rats is that they are being preferentially removed by apoptosis. However, the present study provides no evidence for this theory, because there was no difference in the proportion of late thymic emigrants in the non-viable cell region. The main advantage of using flow cytometry to measure cell death is that cell surface markers can be included to determine the phenotype of the cells that are non-viable. In the present study we used change in light scatter as a marker of cell death; however, inclusion of DNA-binding dyes (i.e. DAPI) to identify cells in the sub G1 area of the cell cycle (DNA fragmentation) or annexin-V (binds to phosphatidylserine; externalisation of phosphatidylserine is an early indicator of apoptosis) would provide additional markers to separate necrotic, apoptotic and viable cells[5]. The methods used in the present study also only offer a snapshot of the splenocytes at a given time; however, an examination of changes over time would be of interest to determine whether the kinetics of cell death are altered by dietary Zn deficiency.

The rat model offers the unique opportunity to identify recently released thymocytes in the periphery using CD90 expression[2]. ZD rats have fewer new splenic T-cells (Fig. 1 (a)); however, by including CD45RC and RT6.1 labels we were able to determine that proportionally the generation of new T-cells by the thymus does not appear to be affected (no difference in the proportion of the most recently released thymocytes) (Table 1). The ZD rat has fewer total T-cells compared with control[6], therefore, the absolute number of T-cells generated by the ZD thymus is undoubtedly lower than the control thymus; however, the proportion of the newest splenic T-cell phenotypes is maintained. Future studies should investigate the kinetics of thymocyte export to determine if output is maintained or slowed down during deficiency.

The expression of CD90 in peripheral lymphoid organs other than blood and spleen has not been explored in a model of dietary Zn deficiency. Perhaps in Zn deficiency the $CD90^+$ cells are homing to other organs such as the lymph nodes or the Peyer's patches instead of the spleen; this needs to be addressed in future studies.

We also report for the first time that growing ZD and DR rats have a lower proportion of new B-cells in the spleen (Fig. 1a). These cells represented a higher proportion of cells in the non-viable cell region in ZD rats compared with control and MZD rats, which provides a possible explanation for their reduced proportion in the viable gate. It remains to be seen if the lower proportion of new B-cells is also due in part to decreased lymphopoiesis in the bone marrow of growing ZD and DR rats or solely post-marrow events. In the bone marrow of ZD adult mice, reports have shown a lower proportion of pre B-cells ($CD45R^+CD43^-IgM^-$), but not the immature plus mature B-cells ($CD45R^+CD43^-IgM^+$)[7].

We found a higher proportion of granulocytes in ZD rats, which is consistent with increased proportions of granulocyte precursors in the bone marrow of adult ZD mice[7]. It is interesting to note that the higher proportion of granulocytes is only seen in the ZD and not in the DR or MZD animals, indicating that reduced feed intake alone or a marginal deficiency of Zn is not sufficient to increase the proportion of granulocytes. It has been suggested that the elevated serum corticosterone concentrations associated with Zn deficiency leave lymphocytes more susceptible to apoptosis while extending the half-life of granulocytes[8]. However, in the present study, we did not find evidence of increased splenocyte susceptibility to cell death, and the proportion of splenic granulocytes in the DR group was not greater despite the fact that serum corticosterone levels in this condition are similar to those in Zn deficiency[6].

The lower proportion of late thymic emigrants and new B-cells in the spleens of ZD and DR rats suggests that there are fewer new T- and B-cells that are able to enter the mature lymphocyte pool. If there are fewer new cells entering the mature lymphocyte pool, then over time the lymphocyte repertoire will become limited, potentially altering T- and B-cell function and the ability of the whole organism to fight off disease[9].

References

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