

**Early thymic T cell development in young transgenic mice over-expressing human Cu/Zn Superoxide Dismutase, a model of Down's syndrome.**

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**Running title:** Early thymic development in SOD1 transgenic mice

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**ABSTRACT**

Previous studies have shown that transgenic mice over-expressing Cu/Zn Superoxide dismutase, a model of Down's syndrome, exhibit premature thymic involution. We have performed an extensive multiparametric flow cytometry analysis of the developing thymus in these homozygous transgenic mice (hSOD1/hSOD1: Tg-SOD). Longitudinal follow-up analysis from day 3 to day 280 showed an early thymic development in Tg-SOD mice as compared with controls. This thymic early development was associated with an increased migration of mature T cells to peripheral lymphoid organs. BrdU labeling showed no difference between Tg-SOD and control mice, confirming that the greater number of peripheral T cells in Tg-SOD mice was not due to extensive proliferation of these cells but rather to a greater pool of emigrant T cells in Tg-SOD.

**Keywords:** Cu/Zn Superoxide dismutase (SOD1), Down's syndrome, Lymph nodes, Reactive oxygen species (ROS), Spleen, Thymus, Transgenic, T lymphocytes,

## INTRODUCTION

The human Copper/Zinc (Cu/Zn)-dependent SuperOxide Dismutase (hSOD1) enzyme catalyses the dismutation of  $O_2$  to  $H_2O_2$  and  $O_2^-$ . The hSOD1 gene is located on chromosome 21 in q22.1 and the SOD1 enzymatic activity is consequently over-expressed in trisomy 21, or Down's syndrome (DS) persons [1, 2]. DS is associated with humoral and cellular immunological abnormalities [3-6], which are more pronounced in aged patients [7, 8]. Moreover altered histology of the lymphoid and epithelial compartments of the thymus has been widely demonstrated [9-12]. Most typical changes in DS thymus include elimination of lymphocytes from the cortical and medullar areas, formation of giant cystic thymic bodies with necrosis, and hyper- or hypoplasia of the reticuloepithelium. In some reports, these changes are considered as the primary cause of immunodeficiency associated with reticuloepithelial pathology, while in others, they rather are considered as secondary manifestations of increased thymic involution which is a complex age-related process [13-17].

It has been proposed that thymic involution is due to alterations in intrathymic T-cell development although changes primarily affecting stromal elements of the thymus cannot be excluded [18]. To explore the possible role of SOD1 over-expression in the premature thymic involution and immune disorders observed in DS patients, transgenic mice over-expressing the human SOD1 gene (hSOD1 Tg mice) were generated [19]. Transgenic mice overexpressing SOD1 has been shown to have thymic abnormalities [20-22]. More recently the Ts65Dn mice, modeling human trisomy 21, has been shown also to have abnormal thymic apoptosis properties in which reactive oxygen species are involved [23]. The early stages of thymic development represent crucial steps for the production of mature T cells [24, 25]. In neonatal and young adult mice, the thymus considerably increases in size and is associated with significant proliferation of both thymocytes and the supporting stromal elements. Thus

thymic dysfunction in hSOD1 Tg mice can be either due to the results of primary immune deficiency or early senescence. [26-29]. As early senescence might be the result of premature T cell differentiation, we have studied the early steps of T cell differentiation both in thymus and peripheral organs of transgenic mice for the human SOD1 gene (Tg-SOD). In this context, we performed an extensive analysis in the thymus and peripheral organs of T lymphocyte development: double negative (DN) cells, which expressed neither CD4 nor CD8 antigen, double positive (DP) cells, which expressed both CD4 and CD8, and simple positive (SP) cells, which expressed either CD4 or CD8. Thymocytes differentiate from DN to DP and then SP cells. At the DP stage, the TCR composed of an alpha/beta heterodimer is expressed for the first time in association with CD3 [30-32]. Up-regulation of the CD69 antigen expression can be used as an indicator of positively selected (DP) cells on the way to single positive cells [33-35].

Precise analysis by multi-parameter flow cytometry showed that early thymocyte differentiation was altered in Tg-SOD mice and was associated with an increased migration of mature T cells to peripheral lymphoid organs.

## **MATERIALS AND METHODS**

### **Mice**

Thymus, spleen and lymph nodes were obtained from transgenic (Tg) mice over-expressing, on the FVB/N strain background, the human superoxide dismutase Cu/Zn (hSOD1) gene including its own promoter (KT line, 21). All the Tg mice used in the present study were homozygous for the hSOD1 transgene (Tg-SOD). Non-transgenic mice (ntg) FVB/N used as controls were obtained in our own animal facilities. Animals aged from 3 to 280 days were maintained under specific pathogen-free conditions in the animal facility of the DRDC/CEA-

Grenoble. The entire research protocol adhered to the guidelines of the European Council directive (86/609/EEC).

### **Superoxide dismutase activity**

For SOD activity, thymus cells were washed three times, resuspended in 10ml of isotonic, trace element-free Tris-HCl buffer (400mM, pH 7.3), and lysed in hypotonic Tris-HCl buffer (20mM) by 5 cycles of freezing and thawing. After 10 minutes of centrifugation at 4,000 rpm, 4°C, the lysate was assayed for metalloenzyme activities and soluble protein content. Total SOD activity corresponding to the sum of the Manganese (Mn)-dependant SOD (SOD2) and the Cu/Zn SOD (SOD1) activities, was determined spectrophotometrically (320nm) using pyrogallol assay [36]. SOD2 is determined by blocking specifically the SOD1 activity with KCN; the difference between total SOD and SOD2 activities allow to determine the SOD1 activity. The amount of SOD inhibiting the reaction rate by 50% in the given assay conditions was defined as one SOD unit. Each sample was analyzed in duplicate, and results were expressed as SOD units and normalized to cell protein content.

### **Flow cytometry analysis**

Cells were stained at  $1 \times 10^6$  cells in 50 $\mu$ l staining buffer (PBS, 1% FBS, 0.01% NaN<sub>3</sub>) so-called FACSwash, and incubated for 15 min at 4°C in the wells of round-bottom 96-well microtiter plates containing saturating concentrations of labeled monoclonal antibodies (mAb) in 150 $\mu$ l FACSwash. Labeled mAb CD8<sup>FITC</sup>, CD8<sup>cyochrome</sup>, CD4PE, CD4<sup>cyochrome</sup>, CD3Cychrome, TCR $\alpha\beta$ <sup>biotin</sup>, CD69<sup>biotin</sup> (PharMingen, San Diego, CA, USA). Following washing in FACSwash, biotin-labeled antibodies were revealed with APC-conjugated streptavidin (SA<sup>APC</sup>) (PharMingen). Cell samples were analyzed by three or four color flow cytometry using a FACSCalibur instrument (Becton-Dickinson, Becton-Dickinson, Le Pont

de Claix, France) and data analyzed using FCSExpress software (Denovo software, Ontario, Canada). For each sample at least  $3 \times 10^5$  cells, gated on a combination of forward (FSC) and side (SSC) scatter, were acquired.

### **BrdU labeling**

One month-aged mice were given 1 mg BrdU i.p. and subsequently given BrdU in their drinking water 1 mg/ml. Cells stained with anti-CD8<sup>cyochrome</sup>, anti-CD4<sup>PE</sup> monoclonal antibodies (PharMingen) were fixed, permeabilized and stained with FITC-labeled anti-BrdU antibody using the BrdU Flow Kit (PharMingen) according to the manufacturer's instructions. Cell samples were analyzed by three color flow cytometry using a FACSCalibur instrument.

### **Cytokine production**

Analysis of individual cytokine-producing cells within mixed populations was carried out with the intracellular cytokine staining kit from PharMingen according to the manufacturer's instructions. Briefly, spleen cells from 1-month-old mice were suspended in D-MEM at  $1 \times 10^6$  cells in 24-well plates, and stimulated for 4 hours with anti-CD3 monoclonal antibody in the presence of the intracellular protein transport inhibitor Brefeldin-A (GolgiStop from PharMingen). Harvested cells were then washed and incubated in 50  $\mu$ l of staining buffer in 96-well plates containing a mixture of anti-CD8<sup>FITC</sup> and anti-CD4<sup>cyochrome</sup> mAb for 15 min at 4°C. After staining cells were washed, fixed and permeabilized with 100  $\mu$ l of Cytotfix/Cytoperm<sup>TM</sup> solution. Permeabilized cells were then washed and suspended in Perm/Wash<sup>TM</sup> solution for 15 min and stained for intracellular cytokines using a cocktail of PE conjugated anti-cytokine antibodies, IL-2<sup>PE</sup>, IL-10<sup>PE</sup>, IFN- $\gamma$ <sup>PE</sup> and TNF- $\alpha$ <sup>PE</sup>. After a final wash, cells were analyzed by three color flow cytometry using a FACSCalibur instrument.

## RESULTS

### *Activity of SOD1 in the Tg-SOD mouse thymus.*

Previous studies with hemizygous hSOD1 Tg (hSOD1/+) mice more than 6 months-old from the same line (KT) indicated an early involution of the thymus architecture [20, 21]. However, no information was yet available concerning the putative effects of the hSOD1 on thymic development in young mice. In preliminary experiments, we measured the activity of total SOD1 activity in the thymus of non-transgenic control and transgenic mice as previously described [36]. In subsequent studies, we used only homozygous hSOD1 transgenic (Tg-SOD) and non-transgenic from the same littermate as control (ntg) mice as controls on the same inbred background (FVB/N). Enzymatic SOD1 activity assays were performed in Tg-SOD mice at 1 and 3 months of age (data not shown). At 1 month of age, the SOD1 activity was five times greater in homozygous Tg-SOD mice ( $102 \pm 20$  U/mg of protein) than in ntg controls ( $22 \pm 6$  U/mg of protein). At 3 months of age, SOD1 activity was decreased but still higher in Tg-SOD ( $58 \pm 10$  U/mg of protein) than in ntg ( $15 \pm 11$  U/mg of protein) mice.

### *Early transient hypertrophy of the Tg-SOD mouse thymus*

Thymus cellularity in Tg-SOD mice was determined by counting viable thymus cells in mice from 3 to 280 days of age. As shown in table 1, the thymus of Tg-SOD was larger than controls ( $30 \times 10^6$  versus  $7 \times 10^6$  at day 3 and  $159 \times 10^6$  versus  $58 \times 10^6$  at day 84). In both Tg-SOD and ntg, thymus cellularity reached a maximum at day 21 ( $563 \times 10^6$  in Tg-SOD versus  $245 \times 10^6$  cells in ntg). From day 21, thymus cellularity decreased significantly in both animals, reaching relatively stable numbers after 84 days in ntg and after 140 days in Tg-SOD ( $48 \times 10^6$  versus  $49 \times 10^6$  at 140 days); until this latter time point, the thymus in Tg-SOD mice was always larger than in ntg. After 140 days the cellularity was stable and similar in both types of mice ( $39 \times 10^6$  versus  $35 \times 10^6$  at 280 days). To compare relative thymic development in Tg-

SOD versus ntg mice, we normalized the maximum thymic cellularity at day 21 to 100% in both mice. Thymus growth was accelerated in Tg-SOD reaching 6% (versus 3% in ntg) at day 3, 49% (versus 25% in ntg) at day 6, 87% (versus 50% ntg) at day 14. In older mice, since 140 days, involution of the thymus was more rapid in transgenic than control mice, with thymus cellularity down at containing only 9% of maximum in transgenic versus 22% in ntg controls. In conclusion, thymus of Tg-SOD mice as compared with control mice, displayed a hypertrophy due to an accelerated growth in the first three weeks of live, which regressed more rapidly with age.

#### *Thymocyte differentiation in Tg-SOD mouse thymus*

In order to determine which steps of the thymus development were affected; thymocytes were harvested at days 3, 6, 14, 21, 84 and 140, and were analyzed for the expression of differentiation markers by the mean of mAb specific for CD8<sup>FITC</sup>, CD4<sup>PE</sup>, CD3<sup>CY</sup> and TCR $\beta$ <sup>APC</sup> or CD69<sup>APC</sup>. At day 3, there was an increase of about 1.8 times in the proportion of both CD4<sup>+</sup> and CD8<sup>+</sup> SP in Tg-SOD, with 12.6 $\pm$ 1.2% and 1.5 $\pm$ 0.4% respectively (Fig 1A, upper left), as compared to ntg, with 7.2 $\pm$ 0.5% and <1% respectively (Fig 1A, down left). At day 21, when thymus size is maximum for Tg-SOD and control mice, these values, reached 21.5 $\pm$ 0.8% versus 12.1 $\pm$ 0.9% for CD4<sup>+</sup> cells and 4 $\pm$ 0.4% versus 1.7 $\pm$ 0.6% for CD8<sup>+</sup> cells in Tg-SOD versus ntg mice, (Fig 1A. upper right). Fig. 1 B and C show the numbers of CD4<sup>+</sup> (B) and CD8<sup>+</sup> (C) SP cells in the thymus of mice at different ages. Until day 100, the numbers of both SP cells type were greater in Tg-SOD mice than in ntg. Altogether these data showed that the number of differentiated thymocytes were temporary increased in mice over-expressing human SOD1, even if the ratio CD4/CD8 is almost the same in both types of mice.

*Expression of TCR and activation marker in early thymocyte maturation*

As total SP thymocytes were dramatically increased in Tg-SOD mice as compared with ntg controls, we analysed various steps of thymocyte differentiation. We investigated the DP and SP cells for the expressions of TCR $\beta$  and CD69, which successively expressed during the process of differentiation and cell activation. For DP T cells, the expression of TCR $\beta$  was similar in both Tg-SOD and ntg mice (Figure 2A. upper panel). For instance, at day 21, the proportion of DP cells expressing high level of TCR $\beta$  (indicated by the horizontal bar above the histograms) is  $18.9\pm 2.6\%$  in Tg-SOD mice (grey area) expressed a high level of TCR $\beta$  versus  $6.5\pm 1.5\%$  in ntg (black area). For SP T cells, at day 3,  $88\pm 9.7\%$  of the CD4<sup>+</sup> and  $84\pm 9.7\%$  of the CD8<sup>+</sup> SP T cells in Tg mice expressed a TCR $\beta$  complex versus  $67\pm 7.6\%$  (Figure 2A, left panels) and  $33\pm 9.7\%$  (data not shown due to the low numbers of events in histogram representation) respectively in ntg mice. At day 21, the percent of SP cells expressing TCR $\beta$  reached  $98\pm 0.5\%$  in both Tg-SOD and ntg mice, but the mean of fluorescence intensity (MFI) was higher in Tg-SOD mice (Fig 2A. right panels). It is striking that TCR $\alpha\beta$  was expressed at high level in Tg SP lymphocytes. For instance at day 3, in CD4<sup>+</sup> cells, TCR $\beta$  expression had a MFI of  $503\pm 29$  for Tg-SOD versus  $226\pm 25$  for ntg mice (Figure 2A, left panels), and in CD8<sup>+</sup> cells the MFI was of  $513\pm 18$  (Figure 2A, left panels) for Tg versus  $229\pm 22$  for ntg mice (data not shown). Again at day 21, in CD4<sup>+</sup> cells TCR $\beta$  expression was with a MFI of  $837\pm 27$  for Tg-SOD versus  $610\pm 34$  for ntg, and in CD8<sup>+</sup> cells the MFI was  $730\pm 45$  for Tg-SOD and  $582\pm 34$  for ntg mice (Fig 2A. right panels).

A similar phenomenon was observed for CD69 expression (Fig 2B.). The proportions of DP T cells expressing of CD69 marker were similarly very low in both Tg-SOD and ntg mice (Figure 2B. upper panel). Briefly, at day 3 less than 2% of DP T cells are CD69<sup>+</sup>, then at day 21 about  $7\pm 2.8\%$  of DP express CD69 in Tg-SOD and ntg mice. For SP cells, at day 3,  $42\pm 5.8\%$  of the CD4<sup>+</sup> and  $18\pm 3.4\%$  of the CD8<sup>+</sup> SP cells in Tg-SOD mice were CD69<sup>+</sup>,

versus an average of 2% of both populations in ntg controls. At day 21, CD69 expression had increased in SP cells from both Tg-SOD and ntg mice, therefore in Tg-SOD mice the proportions of SP CD69<sup>+</sup> were greater than in ntg mice. For instance at day 21, 66±16.3% of the CD4<sup>+</sup> T cells from Tg-SOD are CD69<sup>+</sup> versus 60±5.1% in ntg mice, and 47±14.5% of CD8<sup>+</sup> T cells are CD69<sup>+</sup> versus 40±4.3% in ntg mice (Fig 2B, right panels).

Finally, the numbers of CD69<sup>+</sup> cells in DP, SP CD4<sup>+</sup> and SP CD8<sup>+</sup> T cells follow the same overall variation in function of age for both Tg-SOD and ntg mice (Fig2C), reaching a maximum number at day 21. The number of CD69<sup>+</sup> cells was for every experimental point greater in Tg-SOD than in ntg mice until day 100.

In conclusion, the analysis of TCRβ and CD69 expression revealed some differences in the chronology of the differentiation in Tg-SOD versus ntg mice: at day 3 the expression profiles of the markers in transgenic SOD mice were similar to those obtained at day 21 in ntg mice.

#### *Increased number of T cells in the periphery of Tg-SOD mice*

As we showed that thymus differentiation was accelerated in the thymus of Tg-SOD mice, we examined T lymphocyte populations in the periphery. Phenotypes and cell numbers were analyzed for spleen and lymph nodes T lymphocytes.

In the spleen of 3 day old mice, the cellularity was 3 times greater in Tg-SOD than in ntg mice, then after it was almost similar in both types of mice (Table I). The percentages of CD4<sup>+</sup> and CD8<sup>+</sup> cells were dramatically higher in Tg-SOD than in ntg mice until 21 days of age. At day 3 (Fig 3. upper left), 8.6±1.7% of gated cells were CD4<sup>+</sup> and 3.5±0.8% CD8<sup>+</sup> respectively in Tg-SOD mice. This contrasted with those of 1.2±0.1% and 0.3±0.03% obtained in ntg mice (Fig 3. lower left). Moreover, there were 20 times more CD4<sup>+</sup> (3.9 versus 0.2x10<sup>6</sup>) and 40 times (1.6 versus 0.004x10<sup>6</sup>) more CD8<sup>+</sup> cells in transgenic versus controls. At day 6, total spleen cellularity was roughly similar (58.9 versus 48.7x10<sup>6</sup> cells) in

Tg-SOD and ntg controls but the population of CD4<sup>+</sup> and CD8<sup>+</sup> cells were still higher for transgenic with 14±1.5% versus 0.9±0.06% for CD4<sup>+</sup> and 4±0.7% versus 0.15±0.01% for CD8<sup>+</sup>. At day 14, the percentage increased and remained higher in Tg-SOD mice with 21±1.7% versus 10.1±0.3% for CD4<sup>+</sup> and 5.4±0.7% versus 3.2±0.4% for CD8<sup>+</sup>. At day 21, the percentage of CD4<sup>+</sup> and CD8<sup>+</sup> present in spleen of Tg-SOD mice (Fig 3. upper right) increased to 45±1.9% (versus 31±0.5%) and 11±0.8% (versus 9±0.8%) (Fig 3, lower right). At day 84, there was no difference in the numbers and repartition of T cells in the spleen of Tg-SOD and ntg mice (data not shown).

Lymph node (LN) cell numbers were always greater in Tg-SOD when compared to ntg mice (3-fold at day 14 and 2 fold at day 21 and 84) (Table I.). At day 6, lymph nodes from Tg-SOD mice contained 11.5x10<sup>6</sup> cells, of which 63±1.6% were CD4<sup>+</sup> and 25±1.1% CD8<sup>+</sup>. For ntg mice, it was not possible to collect LN at day 6, because of their too tiny size. At day 14, the cellularity of the LN was 3 times greater in Tg-SOD mice (37.5 versus 12.3x10<sup>6</sup> cells) with 55±4.1% (versus 63±1.5%) CD4<sup>+</sup> and 23±1.2% (versus 21±0.5%) CD8<sup>+</sup> (data not shown). Interestingly, the cellularity and T lymphocyte subset distribution in ntg mice at 14 days were similar to those observed in 6 day old Tg-SOD mice. At day 21, the cellularity of LN increased to 86.7x10<sup>6</sup> (versus 40.7) in Tg-SOD mice whereas the proportions of T cell subsets were similar in both mice, with an average of 45±4.7% for CD4<sup>+</sup> and 20±2.3% for ntg mice. From day 21 to day 84, LN cellularity decreased to reach 34.4x10<sup>6</sup> (versus 15.7) in Tg-SOD mice.

Altogether, the number of T lymphocytes in the periphery is significantly increased in Tg-SOD mice. This augmentation occurred as early as day 3 of life.

*Identical proliferation and activation properties of T cells in Tg-SOD mice*

We seek for difference in the distribution of proliferating thymocytes or peripheral T cells between one month old Tg-SOD and ntg mice by 4 and 48 hours BrdU labeling experiments. After 4 hours of BrdU labeling, a mean of 2.3% of CD4<sup>+</sup> and CD8<sup>+</sup> respectively in Tg-SOD and ntg thymus were BrdU<sup>+</sup> (Table II) and a mean of 0.5% in LN (not shown). After 48 hours, the percentage of BrdU<sup>+</sup> cells increased in both Tg-SOD and ntg thymus to reach 11.4%  $\pm$ 1.8 in Tg-SOD versus 9.7%  $\pm$ 4.6 in ntg mice for CD4<sup>+</sup> SP and 8.3%  $\pm$ 1.2 in Tg-SOD versus 9.4%  $\pm$ 3.4 in ntg mice for CD8<sup>+</sup> SP. In LN, the percentage reached 4.3% in Tg-SOD versus 2.4% in ntg mice for CD4<sup>+</sup> SP and 2.5% in Tg-SOD versus 1.8% in ntg mice (not shown). This analysis showed that whereas the percentage of BrdU<sup>+</sup> cells remained similar in Tg-SOD mice and ntg control thymus or lymph nodes, the total numbers of BrdU<sup>+</sup> cells were greater in Tg-SOD mice, including CD4<sup>+</sup> and CD8<sup>+</sup> SP in both organs.

We further investigated functions of peripheral T cells by measuring their capacity of cytokine productions after anti-CD3 antibody stimulation. After 4 hours of culture, cells were stained for CD8<sup>FITC</sup> and CD4<sup>cy</sup>, fixed and stained intracellular for IL-2, IFN- $\gamma$ , IL-10 and TNF- $\alpha$  using PE-labeled mAbs. The percentages of IL-2 producing cells were lower in Tg-SOD, 36%  $\pm$ 10 for CD4 cells and 18%  $\pm$ 2 for CD8 cells, as compared to ntg mice, 51%  $\pm$ 2 for CD4 cells and 36%  $\pm$ 10 for CD8 cells (Table III). In addition, the MFIs of IL-2 labeling were also lower in Tg-SOD mice with 42  $\pm$ 5 and 37  $\pm$ 6 for respectively CD4 and CD8 cells as compared to 59  $\pm$ 13 and 50  $\pm$ 15 respectively in ntg controls. For IFN- $\gamma$ , the proportion of producing CD8 cells is lower in Tg-SOD, 22%  $\pm$ 2, than in ntg mice, 30%  $\pm$ 2. Staining for IL-10 or for TNF- $\alpha$  did not show any significant difference between the two mice. These data showed that peripheral T cells from Tg-SOD mice have the same capacity of activation than control mice and responded by a duly cytokine production, although IL-2 and IFN- $\gamma$

productions appeared to be statistically decreased, suggesting a reduction in Th1 type of response.

## DISCUSSION

Previous studies report that transgenic mice over expressing Cu/Zn superoxyde dismutase exhibit various immune abnormalities [22]. Homozygous mice from the same transgenic line used in the present study have been shown by electron microscopy to exhibit a premature thymic involution [20, 21]. This thymus premature involution is a characteristic for DS patients [3, 37] and may be related to SOD1 gene dosage since SOD1 is mostly expressed in the human thymic medulla [38]. The involution of the thymus is recognized as the most prominent features of age-related immune senescence [13, 39, 40]. Thymic involution is associated with a gradual decline in the size of the thymus. It has been proposed that thymic involution is due to alterations of the intrathymic T-cell development [41]. We studied by multiparameter flow analysis the T-cell differentiation of Tg-SOD mice versus ntg control littermates in order to evaluate if premature involution reflects a dysfunction of early stages of thymus development. Indeed, we showed that Tg-SOD thymus exhibits a transient atrophy, with an early thymic development (Tab. 1). The colonization of the thymus was shown to happen earlier than in control, following a more rapid decrease from day 140. This observation could explain the earlier thymic architecture disorganization observed in these Tg-SOD mice [21]. Moreover, Tg-SOD mice exhibit productions of both CD4<sup>+</sup> and CD8<sup>+</sup> SP lymphocytes which appear earlier (Fig 1A.) and which are greater along the development until day 100 (Fig 1 B and C) as compared to ntg controls. Several lines of evidence support the fact that differentiation of thymocytes is accelerated in Tg-SOD mice. First, as shown by the profile of TCR $\beta$  expression (Fig 2 A.), mature SP expressing high level of TCR were detected for both CD4<sup>+</sup> and CD8<sup>+</sup> cells in Tg-SOD mice as early as day 3 which is in

accordance with the observation in DS thymus [21]. Second, using CD69 marker as an indicator of positive selection, we showed an earlier activation of both SP in Tg mice (Fig 2B.) and greater numbers of CD69<sup>+</sup> cells in DP and both SP lymphocytes until day 100 (Fig 2C.). Third, it is worth to note that both CD4<sup>+</sup> and CD8<sup>+</sup> SP T lymphocytes follow the same kinetic of production (Fig 1B and C) supporting the idea that the acceleration of differentiation affects similarly both pathways. The BrdU labeling of SP T lymphocytes was similar in Tg-SOD and ntg control mice (Table II), showing that the greater production of SP in thymus of Tg-SOD mice was not due to an extensive proliferation of these cells in Tg-SOD mice. The early maturation of both SP lymphocytes in Tg-SOD mice drives of an early emigration of mature T cells to the periphery which can be detected in spleen as early as day 3 (Fig 3.). The cellularity in lymph nodes showed that the pool of emigrant T cells was greater in Tg-SOD mice (Table I) and followed the profile of T cell production in thymus. The proliferations of T cells in lymph nodes expressing CD4<sup>+</sup> or CD8<sup>+</sup> markers, scored by BrdU labeling, were similar in both Tg-SOD and ntg control mice (data not shown) confirming that the greater number of peripheral T cells in Tg-SOD mice was due to a larger pool of the emigrant T cells in transgenic mice [42-44]. Interestingly, the activity of Cu/Zn SOD decreased after day 21 and was reduced by factor two in 3 month old Tg-SOD mice. It is noteworthy, that cytokine production assays of peripheral T cells reveal few differences between Tg-SOD mice and ntg controls mice, essentially a slight reduction of IL-2 and IFN- $\gamma$  type Th2 cytokine productions. These data are consistent with previous observation showing that over expression of SOD1 does not modify the T cell activation [45].

The early thymic development in Tg-SOD mice and the identical proliferation of SP T lymphocytes in both mice seems to result from alteration in the DP to SP transition. TCR signaling plays a crucial role in regulating thymocyte selection at the DP stage. Thus, the affinity/avidity of TCR-MHC interactions leads to negative selection via induction of

apoptosis. The role of the NF- $\kappa$ B/I $\kappa$ B transcriptional regulatory pathway via TCR signaling in positive or negative selection was demonstrated [46, 47]. Although, NF- $\kappa$ B mediates a survival signal in mature T cells, decreased activity of the NF- $\kappa$ B/Rel pathway results to inhibition of thymic negative selection [48]. Tg mice over-expressing SOD1 down-regulate the NF- $\kappa$ B expression after transient focal cerebral ischemia [49]. It was also reported that mutant SOD1 altered the phosphorylation of I $\kappa$ b, the inhibitor of NF- $\kappa$ B translocation into the nucleus [50]. These data suggested a direct effect of SOD1 activity or expression on modulation of the expression of NF- $\kappa$ B or in the control of NF- $\kappa$ B/Rel pathway. Other data support relationship between reactive oxygen species (ROS) and T cell apoptosis [51]. Indeed, Hidelman and co-workers have shown that ROS can regulate activated T cell apoptosis and T cell responses [52] and that the use of a superoxide dismutase mimetic protects T cell from superoxide generation and cell death [53] therefore suggesting roles for bcl2 and Fas-ligand (FasL). Taking into account all these results, we propose that modulation of Cu/Zn SOD activity affects the negative selection of T cells in Tg mice and subsequently the DP to SP transition. The protective [54-56] or destructive [22, 23, 57, 58] effects of SOD1 overexpression, which remain controversial [59] in the literature might be the result of the apparent antagonist actions of redox balance on separate pathways involved in T cell proliferation, differentiation and apoptosis.

## **CONCLUSIONS**

Our detailed analysis of thymus differentiation reveals alteration of early stages of thymus development in young hSOD1 transgenic mice. As some thymic and more generally immune defects have been also observed in trisomic 16 mice and partial trisomy 16 mice, models of trisomy 21 [23, 60], which contain the SOD1 gene; our results can give some explanations regarding the immunological status of trisomy 21 persons suffering from high rate of

infections, malignancies and some autoimmune disorders which can be explain by immune defects and particularly imbalance of CD4<sup>+</sup> subpopulations. These results are in agreement with the importance of a better understanding of the role of SOD1 overexpression in the immunological status of Down syndrome peripheral lymphocytes as recently shown in a preliminary gene expression profile analysis [61]. A better understanding of the role in the immune system of SOD1 overexpression will be useful for managing the use of pharmacological and/or nutritional anti-oxidant compounds in various pathological conditions.

**List of Abbreviations:**

BrdU, 5-bromo-2'-deoxyuridine; DN, double negative (CD4<sup>-</sup>/CD8<sup>-</sup>) DP, double positive (CD4<sup>+</sup>/CD8<sup>+</sup>); mean fluorescence intensity, MFI; Monoclonal antibody (mAb). Non transgenic control, ntg; SP, simple positive (CD4<sup>+</sup> or CD8<sup>+</sup>); SOD1, copper /zinc superoxide dismutase; Tg-SOD, transgenic mice overexpressing human SOD1.

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**Table I. Cellularity in thymus, spleen and lymph nodes**

Mouse	Age				
<b>Thymus*</b>	<b>3 days</b>	<b>6 days</b>	<b>14 days</b>	<b>21 days</b>	<b>84 days</b>
<b>Tg-SOD</b>	<b>35.6 ±11.6</b>	<b>268.5 ±28.1</b>	<b>474.3 ±19.5</b>	<b>547.6 ±66.5</b>	<b>154.9 ±8.6</b>
<b>ntg controls</b>	<b>7.2 ±1.2</b>	<b>52.2 ±5.2</b>	<b>113 ±5.7</b>	<b>227 ±45.7</b>	<b>58.3 ±21.9</b>
<b>Spleen**</b>	<b>3 days</b>	<b>6 days</b>	<b>14 days</b>	<b>21 days</b>	<b>84 days</b>
<b>Tg-SOD</b>	<b>44.9 ±1.7</b>	<b>58.9 ±2.9</b>	<b>192.5 ±11.8</b>	<b>296.7 ±45.6</b>	<b>144.3 ±14.2</b>
<b>ntg controls</b>	<b>15.7 ±1.6</b>	<b>48.7 ±2.7</b>	<b>175.5 ±22.5</b>	<b>291.7 ±33.6</b>	<b>138.2 ±22.5</b>
<b>Lymph nodes***</b>	<b>3 days</b>	<b>6 days</b>	<b>14 days</b>	<b>21 days</b>	<b>84 days</b>
<b>Tg-SOD</b>	<b>nd****</b>	<b>11.2 ±1.8</b>	<b>37.5 ±2</b>	<b>86.7 ±2.1</b>	<b>34.4 ±2.1</b>
<b>ntg controls</b>	<b>nd</b>	<b>nd</b>	<b>12.3 ±0.6</b>	<b>40.7 ±1.5</b>	<b>15.7 ±2.5</b>

\* Number  $\times 10^6$  cells  $\pm$  S.D, a minimum of 6 independent animals for each value.

\*\* number  $\times 10^6$  cells  $\pm$  S.D, 5 to 6 independent animals for each value.

\*\*\* number  $\times 10^6$  cells  $\pm$  S.D for 10 pooled lymph nodes, 5 to 6 independent animals for each value.

\*\*\*\* nd stands for not done

**Table II. BrdU labeling kinetics of thymocytes**

Mouse		BrdU labeling			
		4 hours		48 hours	
Thymus		<u>SP CD4<sup>+</sup></u>	<u>SP CD8<sup>+</sup></u>	<u>SP CD4<sup>+</sup></u>	<u>SP CD8<sup>+</sup></u>
<b>Tg-SOD</b>	%	<b>2.3 ±0.3</b>	<b>3.3 ±0.2</b>	<b>11.4 ±1.8</b>	<b>8.3 ±1.2</b>
<b>ntg controls</b>	%	<b>2 ±0.3</b>	<b>2 +0.6</b>	<b>9.7 ±4.6</b>	<b>9.4 ±3.4</b>

\* Numbers represent the mean ± S.D of percentages of labeled cells from six experiments using three individual mice.

**Table III. T cells cytokine production test**

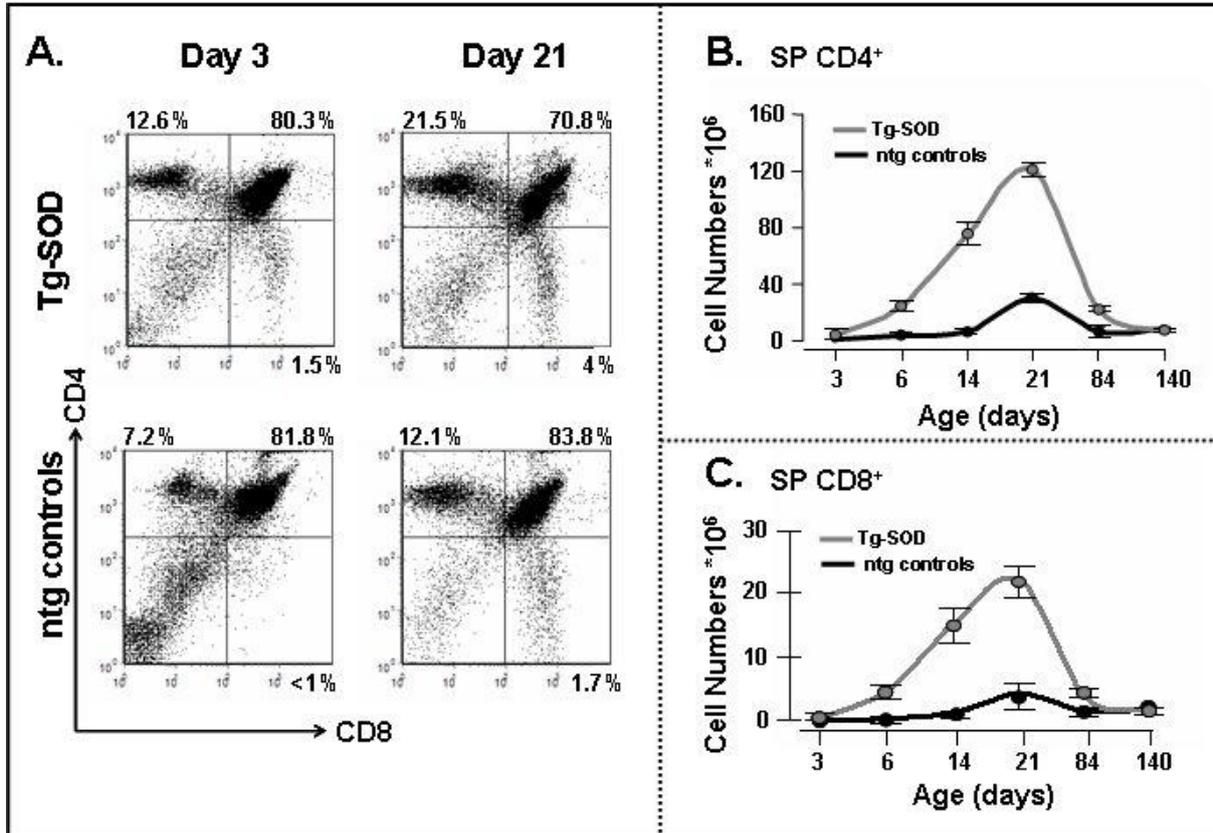
Cytokines		Mouse			
		Tg-SOD		ntg controls	
		CD4 <sup>+</sup>	CD8 <sup>+</sup>	CD4 <sup>+</sup>	CD8 <sup>+</sup>
<b>IL-2</b>	<b>%</b>	<b>36 ±10***</b>	<b>18 ±2***</b>	<b>51 ±2</b>	<b>36 ±10</b>
	<b>MFI</b>	<b>42 ±5**</b>	<b>37 ± 6*</b>	<b>59 ±13</b>	<b>50 ±15</b>
<b>IFN-γ</b>	<b>%</b>	<b>3.7 ±0.2</b>	<b>22 ±2***</b>	<b>5 ±2</b>	<b>30 ±2</b>
	<b>MFI</b>	<b>81 ±21</b>	<b>159 ±31</b>	<b>70 ±13</b>	<b>128 ±54</b>
<b>IL-10</b>	<b>%</b>	<b>2.3 ±0.6</b>	<b>2 ±1</b>	<b>3 ±2</b>	<b>2 ±0.8</b>
	<b>MFI</b>	<b>42 ±7</b>	<b>49 ±5</b>	<b>42 ±10</b>	<b>51 ±4</b>
<b>TNF-α</b>	<b>%</b>	<b>88 ±4</b>	<b>84 ±4*</b>	<b>85 ±6</b>	<b>73 ±11</b>
	<b>MFI</b>	<b>213 ±34**</b>	<b>98 ±8***</b>	<b>173 ±36</b>	<b>77 ±13</b>

Numbers represent average percentages of labeled cells and Mean of Fluorescence Intensity (MFI) ±SD for the corresponding cytokines in three independent experiments using three individual mice.

\*\*\* P ≤ 0.0001; \*\* P ≤ 0.001; \* P ≤ 0.01

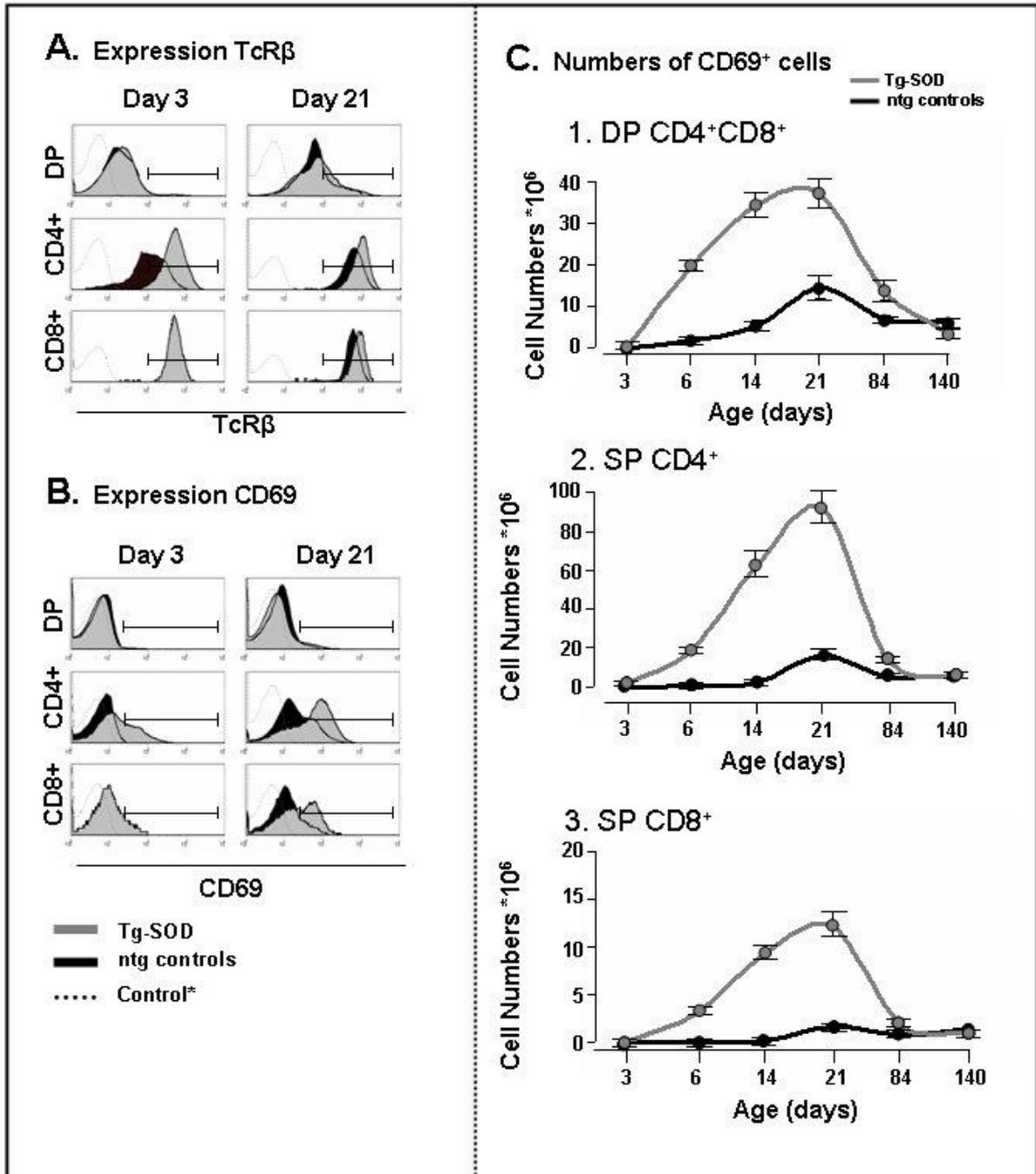
**Figure 1. Phenotype and number of cells in thymus.** A. Phenotype of thymocytes in homozygous Tg-SOD mice (hSOD1/hSOD1) and ntg controls mice were analyzed by two color flow cytometry for expression of CD8 (x axis) and of CD4 (y axis), at day 3 (left panels) and day 21 (right panels). 50,000 events were represented on each dot plot. Percentages of DP, CD4<sup>+</sup> SP and CD8<sup>+</sup> SP are indicated along each relevant area of the plot. B and C. Numbers of CD4<sup>+</sup> SP and CD8<sup>+</sup> SP thymocytes respectively are expressed in millions of thymocytes ( $\times 10^6$ ) for homozygous Tg-SOD mice (open circle) and in ntg control mice (black circle). A minimum of six animals were tested for each point.

Figure 1



**Figure 2. Expression of TCR and CD69 marker.** Expressions of TCR $\beta$  chain (A) or CD69 marker (B) in thymocytes from homozygous Tg-SOD mice (hSOD1/hSOD1) (grey area) and ntg controls mice (black area) were analyzed by flow cytometry at day 3 (left panels) and day 21 (right panels). Dotted line represents the APC-conjugated streptavidin alone as background fluorescence. Horizontal bar indicates areas considered as high expression of markers. C. Numbers of CD69<sup>+</sup> cells in DP, CD4<sup>+</sup> SP and CD8<sup>+</sup> SP thymocytes from homozygous Tg-SOD mice (open circle) and in ntg control mice (black circle) are expressed in millions of thymocytes ( $\times 10^6$ ). A minimum of six animals were tested for each point.

Figure 2



**Figure 3. Phenotype of T lymphocytes in spleen at day 3 and day 21.**

Phenotype of T lymphocytes from spleen of homozygous Tg-SOD mice (hSOD1/hSOD1) (top panels) and ntg control mice(bottom panels) were analyzed by two color flow cytometry for expression of CD8 (x axis) and of CD4 (y axis), at day 3 (left panels) and day 21 (right panels). 50,000 events were represented on each dot plot. Percentages of cells expressing either CD4 or CD8 and of unstained cells are indicated in relevant area of the plot. A minimum of six animals were tested for each point.

Figure 3

