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**Enhanced susceptibility of T lymphocytes to oxidative stress in the absence of the cellular prion protein.**

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**Running title:** PrP<sup>C</sup> and oxidative stress in T lymphocytes

**Abstract**

The cellular prion glycoprotein (PrP<sup>C</sup>) is ubiquitously expressed but its physiologic functions remain enigmatic, particularly in the immune system. Here, we demonstrate *in vitro* and *in vivo* that PrP<sup>C</sup> is involved in T lymphocytes response to oxidative stress. By monitoring the intracellular level of reduced glutathione, we show that PrP<sup>-/-</sup> thymocytes display a higher susceptibility to H<sub>2</sub>O<sub>2</sub> exposure than PrP<sup>+/+</sup> cells. Furthermore, we find that in mice fed with a restricted diet, a regimen known to increase the intracellular level of ROS, PrP<sup>-/-</sup> thymocytes are more sensitive to oxidative stress. PrP<sup>C</sup> function appears to be specific for oxidative stress, since no significant differences are observed between PrP<sup>-/-</sup> and PrP<sup>+/+</sup> mice exposed to other kinds of stress. We also show a marked evolution of the redox status of T cells throughout differentiation in the thymus. Taken together, our results clearly ascribe to PrP<sup>C</sup> a protective function in thymocytes against oxidative stress.

**Key words:**

cellular prion protein

T Lymphocytes

oxidative stress

redox balance

thymus

## INTRODUCTION

PrP<sup>C</sup> is a highly conserved GPI-anchored glycoprotein [1,2] whose true physiological function is still debated [3,4]. Mice lacking PrP<sup>C</sup> are viable and display only minor abnormalities [5,6]. It has been proposed that due to its localization at the cell membrane, PrP<sup>C</sup> could play a role in ligand uptake, cell adhesion or signal transduction [7-11]. Moreover, several groups have shown the ability of this protein to bind copper with high affinity [12-14], ascribing to PrP<sup>C</sup> a role in copper homeostasis. Finally, there is increasing evidence that in the brain, PrP<sup>C</sup> participates in the resistance against oxidative stress [15-18]. It has been shown that neurons from PrP<sup>-/-</sup> mice have an increased susceptibility to superoxide anion and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) toxicity [19,20].

Most studies about PrP<sup>C</sup> are focused on the brain. However, the high expression of PrP<sup>C</sup> is not restricted to the central nervous system [21,22] but is also observed in many non-neuronal tissues including muscles, heart, kidney, hematopoietic cells and cells of the immune system [23,24]. In the hematopoietic compartment, the level of PrP<sup>C</sup> expression is highly variable from one cell type to another and even within a given lineage, depending on the maturation steps [25,26]. For example, it was observed that PrP<sup>C</sup> is differentially expressed on developing thymocytes, suggesting a role of this protein in the regulation of T lymphocyte differentiation [25,27,28].

T cell differentiation in the thymus is a precisely orchestrated process characterized by successive steps which can be followed by the expression of the cell surface molecules CD4, CD8, CD25 and CD44 [29-31].  $\alpha\beta$ T lymphocyte differentiation begins as CD4<sup>-</sup>CD8<sup>-</sup>, double negative (DN) cells. This population is divided into four cell subpopulations based on the regulated expression of CD25 and CD44: CD44<sup>+</sup>CD25<sup>-</sup> (DN1), CD44<sup>+</sup>CD25<sup>+</sup> (DN2), CD44<sup>-</sup>CD25<sup>+</sup> (DN3) and CD44<sup>-</sup>CD25<sup>-</sup> (DN4). Then, DN4 thymocytes progress to CD4<sup>+</sup>CD8<sup>+</sup> double positive (DP) cells and express low levels of  $\alpha\beta$ TCR. A fraction of the DP cells is positively selected to become either CD4<sup>+</sup> or CD8<sup>+</sup> single-positive (SP) cells, which finally exit the thymus and migrate to the periphery. This differentiation is highly influenced by microenvironment conditions. It is well known that the redox system and antioxidant enzymes have an effect on both cell signalization [32,33] and apoptosis mechanisms which occur during T cell differentiation in the thymus [33,34]. *In vivo*, it appears that antioxidant conditions disrupt immature thymocyte development. In thymic organ cultures, antioxidants selectively affect  $\alpha\beta$ <sup>+</sup> thymocyte differentiation via their effects on NF- $\kappa$ B [35] which plays a

crucial role in lymphocyte maturation [36]. Similarly, transgenic mice overexpressing Cu/Zn superoxide dismutase exhibit various immunological abnormalities, including an early thymic involution [37,38].

We previously reported that over-expression of PrP<sup>C</sup> in mice resulted in strong alterations at different stages of T cell differentiation in the thymus. These effects could be partially reversed *in vivo* by copper supplementation in the drinking water, which led us to propose that the over-expression of PrP<sup>C</sup>, by chelating copper, creates an antioxidant context that is not supportive for  $\alpha\beta$  T cell development [28]. In the present study, we investigated the function of the PrP<sup>C</sup> protein in the thymic redox homeostasis. We first compared the response of PrP<sup>-/-</sup> and WT thymocytes in culture to an oxidative stress using H<sub>2</sub>O<sub>2</sub> exposure. Then, to analyze the effects of oxidative stress *in vivo*, mice were submitted to a restricted feeding schedule, known to increase the intracellular level of reactive oxygen species (ROS) [39]. Finally, to assign more precisely to PrP<sup>C</sup> a role in the defense against oxidative stress, we carried out two other types of stress: mice received dexamethasone by IP injection or were submitted to gamma-irradiation. Altogether our results demonstrate that thymocytes from PrP<sup>-/-</sup> mice are much more affected than their WT counterparts by both *in vitro* and *in vivo* oxidative stress. We therefore propose that PrP<sup>C</sup> plays an important role in the protection of thymocytes from oxidative stress, extending to the thymus the role of PrP<sup>C</sup> in the redox homeostasis previously described in the brain.

## MATERIALS AND METHODS

### Mice

C57BL/6 mice were purchased from Charles River Laboratories (l'Arbresle, France). PrP<sup>-/-</sup> mice [40] on a mixed C57BL/6 / 129 background were obtained from the Centre de Distribution, Typage et Archivage animal facility (CNRS, Orléans, France). Both strains were maintained under specific pathogen-free conditions in the animal facility of the Commissariat à l'Energie Atomique-Grenoble (A38 185 01), in accordance with institutional guidelines.

### Cell culture and oxidative stress

Thymocytes were isolated from 4- to 6-week-old C57BL/6 or PrP<sup>-/-</sup> mice and cultured in RPMI 1640 medium supplemented with 10% FCS, 1 mM sodium pyruvate, 1% non essential amino acids, 50  $\mu$ M  $\beta$ -mercaptoethanol, 1% penicillin (100 U/ml), 1% streptomycin (100

µg/ml) (all from InVitrogen Life Technologies, Cergy Pontoise, France). Cells ( $3 \times 10^6$ /ml) were cultured in 24-wells plates (1 ml/well) at 37°C in humidified 5% CO<sub>2</sub> in air. Oxidative stress was induced by addition to the cells of 200 µM H<sub>2</sub>O<sub>2</sub> and incubation for the indicated period of time. Cell viability was determined at the end of the treatment by acridin orange/ethidium bromide staining under UV light.

### **Flow cytometry analysis**

For the analysis of cell surface protein expression, single-cell suspensions of thymocytes ( $1 \times 10^6$ ) were stained for 30 min at 4°C with conjugated antibodies in PBS containing 3% fetal calf serum and 0.16% sodium azide. The antibodies used for staining (anti CD4, anti CD8, anti CD25 and anti CD44) were all from BD Pharmingen (le Pont de Claix, France). Various combinations of these antibodies labeled with FITC (excitation 488 nm, emission 525 nm), PE (excitation 488 nm, emission 575 nm), APC (excitation 633 nm, emission 670 nm) and PE-Cy5 (excitation 488 nm, emission 760 nm) were used. Data acquisition and analysis were performed with a FACS Calibur flow cytometer equipped with CellQuest software (Becton Dickinson).

### **Measurement of intracellular glutathione**

Monochlorobimane (mCB) is a thiol-reactive cell permeable reagent able to bind to the intracellular reduced glutathione (GSH) [41]. The resulting adduct is fluorescent. mCB was added to the cells as described [42]. Briefly, cells were washed twice with PBS and stained with 50 µM mCB (Molecular Probes, Interchim, Montluçon, France) in PBS for 5 min at 37°C. After a further incubation for 5 min on ice, the cellular fluorescence was analyzed by flow cytometry under UV excitation, using a Moflo cytometer (Dako, Trappes, France). In these conditions the level of fluorescence intensity is directly correlated to the intracellular level of reduced glutathione. The numbers provided in Table 1 represent the mean fluorescence intensity ( $\pm$  s.d) of mCB staining in the different thymocyte populations. Because identical results were obtained in WT and PrP<sup>-/-</sup> mice, results from these two groups of animals were grouped for statistical significance analysis (Student's t test, SPSS Software ver 15.0)

### **Measurement of glutathione reductase levels in thymocytes**

Assay of glutathione reductase (GRase) activity was adapted from Carlberg and Mannervik [43]. The principle is based on the measurement at 340 nm of NADPH consumption catalyzed

by GRase during the reduction of GSSH. Results were expressed as mean international units per gram of protein  $\pm$  s.d. of at least 3 experiments. Statistical significance was determined using Student's *t* test.

### **Copper dietary supplementation**

Supplemented C57BL/6 or PrP<sup>-/-</sup> mice were given water containing 250 mg/L CuSO<sub>4</sub> and 50 mg/L sucrose, whereas the control group received only sucrose. The diet was maintained for five weeks and then the mice were sacrificed for analysis of the thymocytes.

### **Restricted feeding experiment**

Mice were kept under a restricted diet consisting in the free access to food and water for only 2 hours per day as described in [39]. After 7 days, diet was stopped and mice had again unlimited access to food and water. Throughout this experiment, 3 to 4 mice were tested every day for their ability to respond to the stress generated by the diet. For this purpose, mice were sacrificed and their thymi recovered. Total numbers of thymocytes were measured and cells were incubated with anti-CD4 and anti-CD8 antibodies for flow cytometry analysis

### **Dexamethasone-mediated apoptosis**

Mice were subjected to two injections of dexamethasone (2 mg/kg; i.p.) separated by 24 hours. They were killed 24 hours after the last injection and thymocytes were analyzed by FACS as described above.

### **Gamma irradiation**

Mice were irradiated (6Gy) in the "Anémone/Bio" irradiator (<sup>60</sup>Co, 2 Gy/min) in the "Arc/Nucléart" facility at the CEA-Grenoble. Mice were sacrificed 24 hrs post irradiation.

## **RESULTS**

### **Measurement of the intracellular redox steady state of T lymphocyte precursors**

Glutathione is implicated in the protection of cells from reactive oxygen species such as free radicals and peroxides: reduced glutathione (GSH) acts as a scavenger of ROS and is regenerated through transient formation of the oxidized form (GSSG). In order to evaluate the



redox state of thymocyte subpopulations, C57BL/6 and PrP<sup>-/-</sup> thymocytes were labeled with anti-CD4, anti-CD8, anti-CD44, and anti-CD25 antibodies and the content in GSH of the different subpopulations was measured by the mCB assay, as described in Materials and Methods. We previously showed that T cell differentiation is normal in PrP<sup>-/-</sup> mice [28]. Overall identical results were obtained by the mCB assay for both types of mice (Fig 1 and Table 1). As shown in Fig 1A for WT mice, the most immature thymocytes (DN1/DN2 cells) contained the highest level of GSH. These cells were uniformly mCB<sup>bright</sup>. The intensity of the staining then decreased as the cells progressed until the DN3 stages ( $p < 0.001$  when data from both types of mice are pooled) and DN4 ( $p < 0.05$  with DN3). For both types of mice (Fig 1A and 1B) there was a further reduction in the intracellular content in GSH when the developing thymocytes reached their DP stage ( $p < 0.001$  between DN4 and DP), whereas a slight increase is noted during the final maturation of thymocytes to the SP stage ( $p = 0.003$  between DP and SP CD4<sup>+</sup> and  $p < 0.001$  between DP and SP CD8<sup>+</sup>). Interestingly, while the staining was homogeneous for the DN1/DN2, DN3, DP and SP CD4<sup>+</sup> populations, the distribution of mCB<sup>+</sup> DN4 cells was broader and likely corresponded to an intermediate between the staining level of their DN3 precursors and their DP progeny. This dichotomy could reflect the transitional nature of the DN4 population, as these cells cycle rapidly while they exit the DN compartment to become DP. Similarly, the staining of the SP CD8<sup>+</sup> cells was biphasic: the vast majority of the cells contained low DP-like levels of GSH while a minor population exhibited a mCB staining intensity similar to that found in DN3. This last population could represent immature SP thymocytes, an intermediate population of cycling cells on their way to become DP. Altogether, these results show that there is a marked evolution of the redox status during normal thymocyte development in mice. The same level of mCB staining is obtained for a given thymocyte population in different mice. (Fig 1B and Table 1). Interestingly, this pattern of intracellular GSH was also found in mutant mice where T cell development is impaired because a lack of pre-TCR expression, pT $\alpha$ <sup>-/-</sup> mice and CD3 $\epsilon$  <sup>$\Delta 5/\Delta 5$</sup>  mice (data not shown), underscoring the regulated control of the redox state during thymocyte differentiation.

### **Thymocytes from PrP-deficient mice are more susceptible to a H<sub>2</sub>O<sub>2</sub> stress**

In order to extend our *in vitro* observations to an *in vivo* context and in order to evaluate whether PrP<sup>C</sup> expression plays a role in the modification of the cellular level of GSH in thymocytes after an oxidative stress, PrP<sup>-/-</sup> and WT cells were incubated with H<sub>2</sub>O<sub>2</sub>. Hydrogen peroxide crosses freely the membrane and induces in the cytoplasm the generation of the

highly reactive hydroxyl radical OH<sup>•</sup>[44]. Thymocytes from C57BL/6 or PrP<sup>-/-</sup> mice were plated as described in materials and methods and incubated for 4 hours in the presence of 0.2 mM H<sub>2</sub>O<sub>2</sub>. At this concentration, which was previously used to analyze the effect of stress on antigen presenting cells [45,46], the cellular viability was not affected (data not shown). In C57BL/6 thymocytes, this treatment resulted in a decrease in the percentage of cells positive for mCB staining (Fig 2A), i.e that do contain high levels of GSH. But this decrease was much more important in absence of PrP<sup>C</sup> expression. The PrP<sup>-/-</sup> mice used for these analyses are on a mixed C57BL/6 / 129 background. Experiments were repeated with mice that were backcrossed more than 10 times to C57BL/6 mice. Similar results were obtained (data not shown), showing that this effect was not due to a strain difference. Thus, in an oxidative stress situation, PrP<sup>-/-</sup> thymocytes are less able to maintain their pool of GSH, showing a difference in the capacity of these cells to respond to H<sub>2</sub>O<sub>2</sub> exposure.

In PrP<sup>-/-</sup> mice, neurons have also been shown to be over sensitive to H<sub>2</sub>O<sub>2</sub> exposure. It was suggested that this effect resulted from a decrease in glutathione reductase (GRase) activity [20,47]. To determine whether the increased susceptibility to peroxide toxicity observed in PrP<sup>-/-</sup> mice was linked to an altered GRase activity, we measured it in WT and PrP<sup>-/-</sup> thymocytes, before and after incubation with H<sub>2</sub>O<sub>2</sub> (Fig 2B). In the absence of peroxide, GRase activity was unchanged in PrP<sup>-/-</sup> thymocytes when compared to WT. This activity was significantly increased in PrP<sup>-/-</sup> but not WT thymocytes in the presence of 0.2 mM H<sub>2</sub>O<sub>2</sub>. These results show that the increased susceptibility of PrP<sup>C</sup>-deficient thymocytes to H<sub>2</sub>O<sub>2</sub> exposure does not result from a reduced GRase activity as this activity was on the contrary increased in this context. This increase was however not sufficient to prevent the decrease of the GSH level in PrP<sup>-/-</sup> thymocytes. In addition, these results show that the GRase activity is differentially regulated in neurons and thymocytes,

Knowing that PrP<sup>C</sup> binds copper and that this cation plays a crucial role in the regulation of the redox balance, we tried to restore, in mice lacking the PrP<sup>C</sup> protein, a correct redox intracellular environment by supplementing their drinking water with copper. The results of this copper supplementation are shown in Fig 3. There was no difference in the content in GSH between WT and PrP<sup>-/-</sup> thymocytes when no stress was applied. Addition of copper did not change the GSH level. On the other hand, while the content of reduced glutathione after H<sub>2</sub>O<sub>2</sub> treatment was not significantly modified in copper supplemented C57BL/6 as compared to non treated mice, copper addition allowed the PrP<sup>-/-</sup> mice to limit the effects of the H<sub>2</sub>O<sub>2</sub> stress: the level of reduced glutathione was significantly increased and reached nearly that of

treated WT mice. This last result reinforces the hypothesis that PrP<sup>C</sup> could contribute to the preservation of the redox homeostasis.

### **Effect of a physiologic oxidative stress on mice**

In order to extend our *in vitro* observations to an *in vivo* context, we exposed thymocytes in their physiological environment to an oxidative stress. To this end, mice were kept under a restricted diet: free access to food and water was allowed only for two hours per day for seven days. This regimen has previously been shown to increase the intracellular level of ROS [39] and to induce thymocyte apoptosis [48,49]. We therefore compared C57BL/6 and PrP<sup>-/-</sup> mice in their ability to respond to this type of stress by evaluating, each day, the thymus cellularity and the distribution of thymocyte populations. As shown in Fig 4A, in treated C57BL/6 mice, thymic cellularity remained constant for the first 4 days of food restriction (between  $216 \times 10^6$  and  $197 \times 10^6$  cells). Then it fell at day 6 to reach a minimum ( $30.10^6$  cells) one day after the end of the restriction period (day 8). By the second day after the return to a normal regimen (day 9), the number of thymocytes already began to increase ( $71 \times 10^6$  cells). In treated PrP<sup>-/-</sup> mice, thymic cellularity was found to decrease already from the first day of the food restriction period ( $131 \times 10^6$  versus  $189 \times 10^6$  cells). This decrease was strong throughout the treatment, so that on the 6<sup>th</sup> day, PrP<sup>-/-</sup> thymus contained only  $12 \times 10^6$  cells, compared to  $85 \times 10^6$  cells in C57BL/6 mice. As for WT mice, this reduction in thymocytes number was prolonged after the end of the treatment: the first and the second days after the return to a normal diet, the thymus of the treated animals contained only about  $2 \times 10^6$  cells. It is only at day 11, four days after the end of the restriction period, that the number of thymocytes began to increase, whereas this trend was already present in WT mice at day 9. Thus, the loss of cellularity began earlier, was much more pronounced and needed a longer period to be reverted in absence of PrP<sup>C</sup> expression. Altogether these results confirm that developing thymocytes are more sensitive to oxidative stress in the absence of PrP.

In parallel, we investigated the consequences of this treatment on each thymocyte subpopulation. FACS analysis revealed that thymocytes at every stage of differentiation were affected. The absolute number of cells in each of the four main compartments (DN, DP, CD4 and CD8) decreased after restricted feeding (Fig 4B). Two days after the end of the treatment, in C57BL/6 mice, the number of cells present in the DN, DP, SP CD4 and SP CD8 compartments were only 25%, 10%, 30% and 48% of the numbers found in control mice, respectively. In PrP<sup>-/-</sup> mice, the consequences of the applied regimen were clearly more severe: only 4.8%, 0.1%, 4% and 6.5% of the cells were left in the DN, DP, SP CD4 and SP CD8 compartments in treated mice, respectively. In addition, even though DP thymocytes are

the most affected cells in the thymus irrespective of PrP<sup>C</sup> expression, in the absence of PrP<sup>C</sup> these cells became a hundred times more sensitive to the oxidative stress generated by food restriction (0.11% vs 10.12% of cells remaining in PrP-deficient and wt mice, respectively). Altogether, these results reveal a higher susceptibility of PrP<sup>-/-</sup> mice to oxidative stress *in vivo*.

### **Effect of non oxidative stress on PrP<sup>-/-</sup> mice**

We then wanted to study whether the absence of PrP<sup>C</sup> had also deleterious effects on developing thymocytes in mice confronted with other types of stress. To this purpose, mice were exposed to two different types of stress. First, C57BL/6 and PrP<sup>-/-</sup> mice were injected with dexamethasone. This glucocorticoid hormone is known to induce thymocyte apoptosis [50]. C57BL/6 and PrP<sup>-/-</sup> mice received two IP injections of dexamethasone (2 mg/kg) separated by 24 hours, then, 24h after the last injection, CD4<sup>-</sup>CD8<sup>-</sup>, CD4<sup>+</sup>CD8<sup>+</sup>, SP CD4<sup>+</sup>, and SP CD8<sup>+</sup> subpopulations were analyzed by flow cytometry. As shown in Table 2, dexamethasone induced an important reduction of the percentage of DP in C57BL/6 and PrP<sup>-/-</sup> thymocytes. This reduction was nevertheless highly comparable between both types of mice. Thus, absence of PrP expression does not influence thymocyte response to dexamethasone injection *in vivo*.

We then used gamma irradiation which is known to strongly affect thymocytes [51-53]. Both PrP<sup>-/-</sup> and C57BL/6 mice were exposed to a single dose (6 Gy) of whole body <sup>60</sup>Co irradiation. DP cells are known to be highly radio-sensitive and, accordingly the percentage of DP was strongly reduced after irradiation, irrespective of PrP<sup>C</sup> expression (Table 2). It varied from 84.11 ± 1.71% for un-irradiated C57BL/6 mice to 17.75 ± 4.69% after irradiation and from 82.32 ± 1.76% to 27.58 ± 14.40% for PrP<sup>-/-</sup> mice. We observed compensatory increases in the other subpopulations after irradiation, but no significant differences between PrP<sup>-/-</sup> and WT mice. These results show that *in vivo* PrP<sup>-/-</sup> thymocytes behave like wild-type cells when exposed to no oxidative stress.

## **DISCUSSION**

In a previous report, we showed that over-expression of the PrP<sup>C</sup> protein at the surface of thymocytes was at the origin of defects in the development of T cell precursors in the thymus, and that copper supplementation induced a partial restoration of T lymphocyte differentiation [28]. These results led us to propose that the over-expression of PrP<sup>C</sup> increases copper

chelation and thus creates an antioxidant environment in the cells that interferes with a correct  $\alpha\beta$  T cell development. The aim of the present study was to determine whether in the absence of PrP<sup>C</sup>, thymocytes are more susceptible to oxidative stress and whether PrP<sup>C</sup> could contribute to the response of thymocytes against oxidative stress. To this purpose we compared *in vitro* and *in vivo* the response of WT and PrP<sup>-/-</sup> thymocytes exposed to different stress. Our results show that thymocytes from mice that do not express the PrP<sup>C</sup> protein are less efficient in their capacity to respond to an oxidative stress as compared to WT mice, but behave like WT thymocytes after radiation exposure or dexamethasone treatment.

Oxidative stress is the result of the imbalance between production and destruction of ROS, leading to modification of the intracellular redox state. This imbalance can result from exposure of the cells to an excess of oxidant species or from a cellular deficit in antioxidant molecules such as glutathione [44]. Reduced glutathione is critical for the prevention of lipid and protein oxidation and the detoxification of ROS. It is essential for the regulation of the redox balance. Thymocyte development and T lymphocyte activities are markedly influenced by environment conditions. For example exposure to ROS has been shown to impair TCR signaling [54] and T lymphocyte activation and functions [55-57]. Conversely, optimal proliferation and activation of mature lymphocytes require reducing conditions [55,58].

Our results now show that each discrete thymocyte subpopulation is characterized by a specific level of reduced glutathione. The highest quantity of GSH is found in the more immature cells and it regularly decreases as the cells become more and more mature, to reach its lowest level in the DP/SP subpopulations. It would be interesting to determine whether this developmental regulation of the intracellular GSH is required for proper thymocyte development or is imposed onto developing thymocytes by their maturation process. This difference of redox state of the various T cell subpopulations may be used as a marker to characterize the progress in the differentiation pathway.

Glutathione is only one of the components participating in the regulation of intracellular redox balance in thymocytes. By chelating copper, the PrP<sup>C</sup> protein could also play a role in this process but it appears dispensable in normal conditions, as similar levels of GSH are found in C57BL/6 and PrP<sup>-/-</sup> cells. However, when thymocytes are exposed to oxidative stress conditions, a marked difference between C57BL/6 and PrP<sup>C</sup>-deficient mice becomes evident: GSH is strongly diminished and/or not rapidly replenished in PrP<sup>-/-</sup> thymocytes. In PrP<sup>-/-</sup> neurons, it has been shown that this effect was the result, at least in part, of the low glutathione reductase activity [20]. Obviously, this is not the case in lymphocytes, as we found a higher GRase activity in PrP<sup>-/-</sup> thymocytes after the H<sub>2</sub>O<sub>2</sub> stress. It seems that after

H<sub>2</sub>O<sub>2</sub> exposure, these PrP-deficient thymocytes enhanced their GRase activity in an attempt to preserve a correct GSH pool but this increase in GRase activity cannot maintain/restore a normal level of intracellular GSH in PrP<sup>-/-</sup> thymocytes. Thus, paradoxically, the absence of PrP<sup>C</sup> results in a lower level of GSH and a greater GRase activity in response to oxidative stress, demonstrating the importance of the PrP<sup>C</sup> protein in the regulation of the GSH pool and the redox homeostasis.

Interestingly, we can note a difference within mice that have received a copper supplementation: for WT mice the content of GSH in thymocytes after exposure to H<sub>2</sub>O<sub>2</sub> is not changed as compared to non supplemented mice, whereas addition of copper allows the cells from PrP<sup>-/-</sup> mice to face the oxidative stress, having a GSH content much higher than thymocytes from mice that have not been treated with copper. This result strengthens the hypothesis that PrP<sup>C</sup> could play a role in redox homeostasis. We can thus conclude that the function of the PrP<sup>C</sup> protein is revealed under oxidation stress conditions *in vitro*.

Similarly, in living mice, the loss of PrP<sup>C</sup> is also correlated with an increased susceptibility to oxidative stress generated via food restriction. The decrease in total thymic cellularity is more pronounced and the effect lasts for a longer period for PrP<sup>-/-</sup> mice as compared with WT mice. In both mice, food deprivation strongly affects DP cells but this effect is much more pronounced in the case of PrP<sup>-/-</sup> mice. We propose that, due to its capacity to bind copper, PrP<sup>C</sup> participates to the fine regulation of the redox balance and consequently contributes to the regulation of thymocyte differentiation.

PrP<sup>C</sup> has been shown to be implicated in many processes where redox regulation is involved: in particular, during antigen-driven interactions PrP<sup>C</sup> appears to be mobilized at the immune synapse [59]. PrP<sup>C</sup> is associated with lipid rafts and it has been well documented that cross-linking of PrP<sup>C</sup> on mature T lymphocytes leads to PrP<sup>C</sup> capping in clusters also containing Thy-1 and molecules implicated in T cell activation, i.e., TCR/CD3, ZAP70, Fyn, Lck and LAT [60-62]. It has been recently reported that oxidative stress results in oxidation of the actin-remodelling protein cofilin [63]. Oxidation of this key integrator of T cell activation leads to impaired actin depolymerisation and results in T cell hyporesponsiveness under stress conditions due to incomplete immune synapse formation [63]. Antibody-mediated ligation of PrP<sup>C</sup> at the surface of T lymphocytes promotes NADPH oxidase activation and a transient accumulation of ROS which act as second messenger, through phosphorylation of MAPK-ERK1/2 [10]. In this case ROS are no longer considered as responsible for an oxidative stress but rather as key actors of intracellular signalization [64]. The activation of ERK by NADPH

oxidase products, in response to PrP<sup>C</sup> ligation, could allow the preservation of the redox status of the cell. Our results show that both *in vivo* and *in vitro*, the response of thymocytes to oxidative stress is altered in the absence of PrP<sup>C</sup>. Taking into account published data and our present study, we propose that in the absence of PrP<sup>C</sup>, the signaling pathway which leads to the phosphorylation of the ERK1/2 kinases, is disturbed, with no direct consequences in normal conditions but leading to the disruption of the oxidant/antioxidant balance in case of a redox stress.

Our results underline the importance of the redox balance in the different steps of T cell differentiation. Moreover, by *ex vivo* and *in vivo* experiments, we have demonstrated that in the absence of PrP<sup>C</sup>, thymocytes are less able to face an oxidative stress and to maintain their intracellular GSH level. Altogether, our results show that PrP<sup>C</sup> have a protective function against oxidative stress and in the preservation of the intracellular redox balance.

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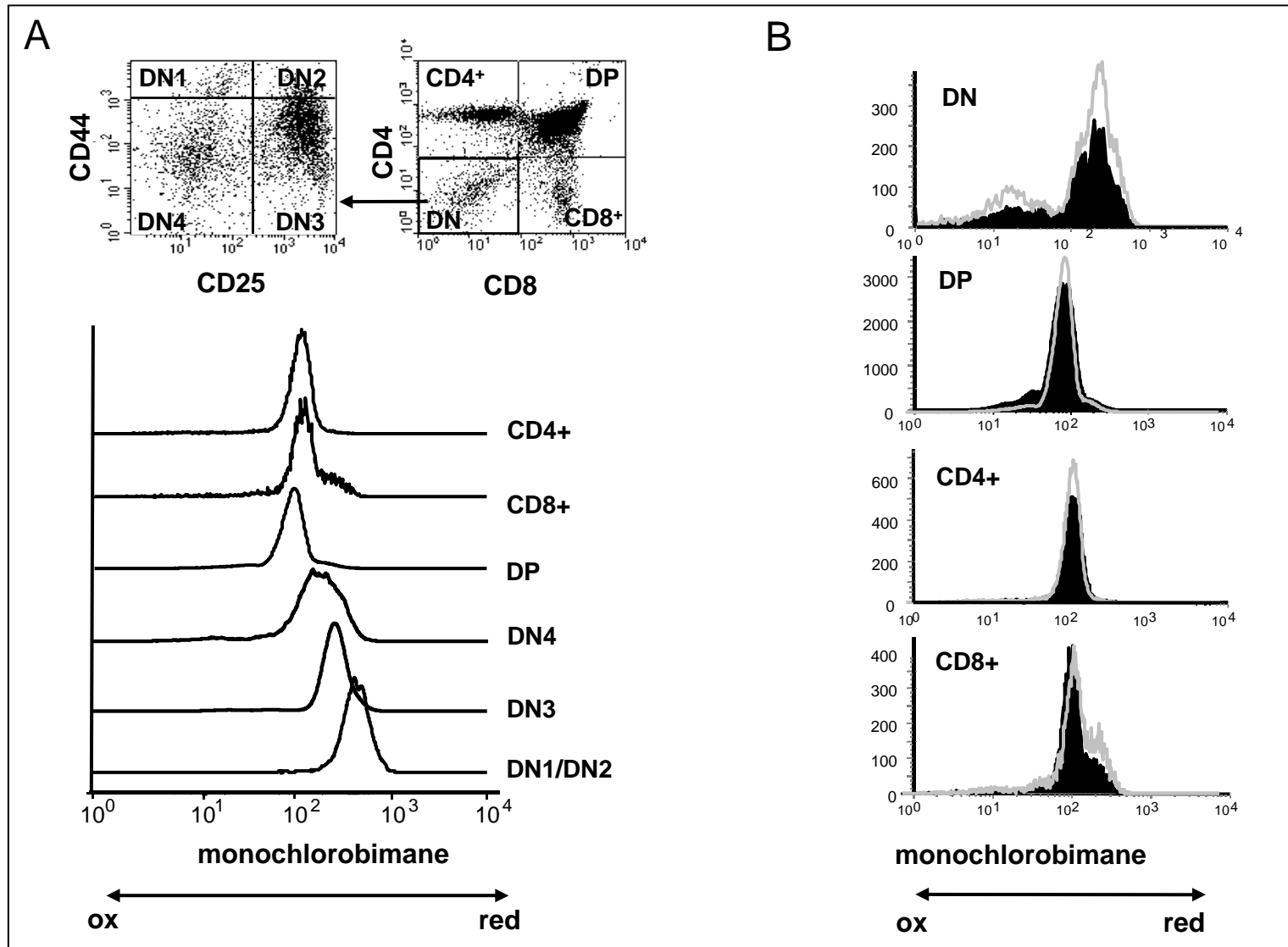
**Table 1:** reduced glutathione level in the different thymic subpopulations<sup>a</sup>. The results are expressed as mean fluorescence units  $\pm$  s.d of monochlorobimane staining intensity (n=3)

	DN1-DN2	DN3	DN4	DP	CD4	CD8
C57BL/6 $\pm$ s.d	3112 $\pm$ 80	1968 $\pm$ 41	2053 $\pm$ 108	629 $\pm$ 23	725 $\pm$ 57	883 $\pm$ 53
PrP <sup>-/-</sup> $\pm$ s.d	3125 $\pm$ 73	2077 $\pm$ 151	2150 $\pm$ 125	662 $\pm$ 42	760 $\pm$ 85	1003 $\pm$ 118

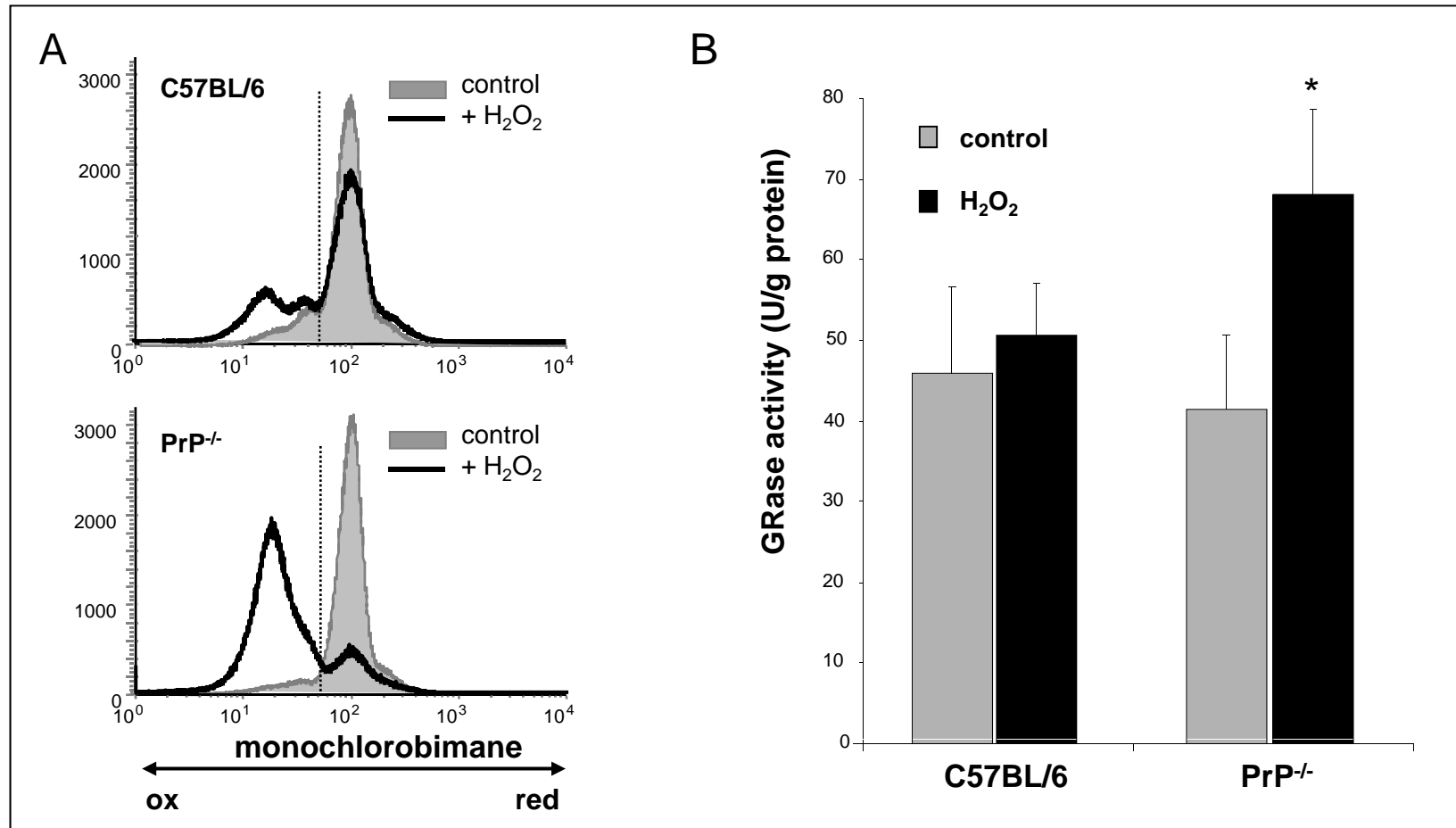
<sup>a</sup>: the thymic subpopulations were defined as described in Figure 1

**Table 2:** effect of dexamethasone and  $\gamma$ -irradiation on the different thymic subpopulations in PrP<sup>-/-</sup> and C57BL/6 mice. n  $\geq$  5.

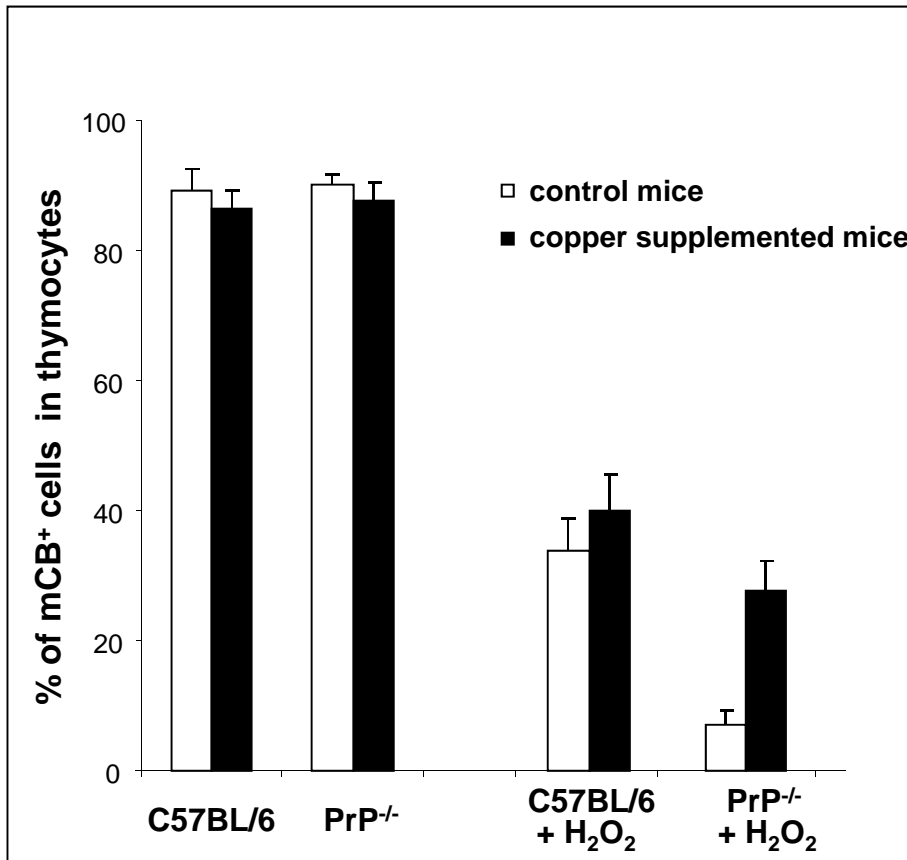
Mice	% DN $\pm$ s.d.	% DP $\pm$ s.d.	% CD4 <sup>+</sup> $\pm$ s.d.	% CD8 <sup>+</sup> $\pm$ s.d.
C57BL/6 control	3.50 $\pm$ 0.42	84.11 $\pm$ 1.71	7.67 $\pm$ 0.65	2.54 $\pm$ 0.49
PrP <sup>-/-</sup> control	3.36 $\pm$ 0.53	82.32 $\pm$ 1.76	8.61 $\pm$ 0.45	3.32 $\pm$ 0.77
C57BL/6 + dexamethasone	16.01 $\pm$ 0.94	6.36 $\pm$ 0.80	29.44 $\pm$ 0.30	16.43 $\pm$ 1.00
PrP <sup>-/-</sup> + dexamethasone	12.24 $\pm$ 1.05	8.45 $\pm$ 3.28	30.88 $\pm$ 2.21	17.88 $\pm$ 1.23
C57BL/6 6 Gy	20.39 $\pm$ 1.30	17.75 $\pm$ 4.69	43.41 $\pm$ 10.61	16.76 $\pm$ 3.28
PrP <sup>-/-</sup> 6 Gy	17.87 $\pm$ 4.45	27.58 $\pm$ 14.40	40.21 $\pm$ 7.29	14.34 $\pm$ 3.01



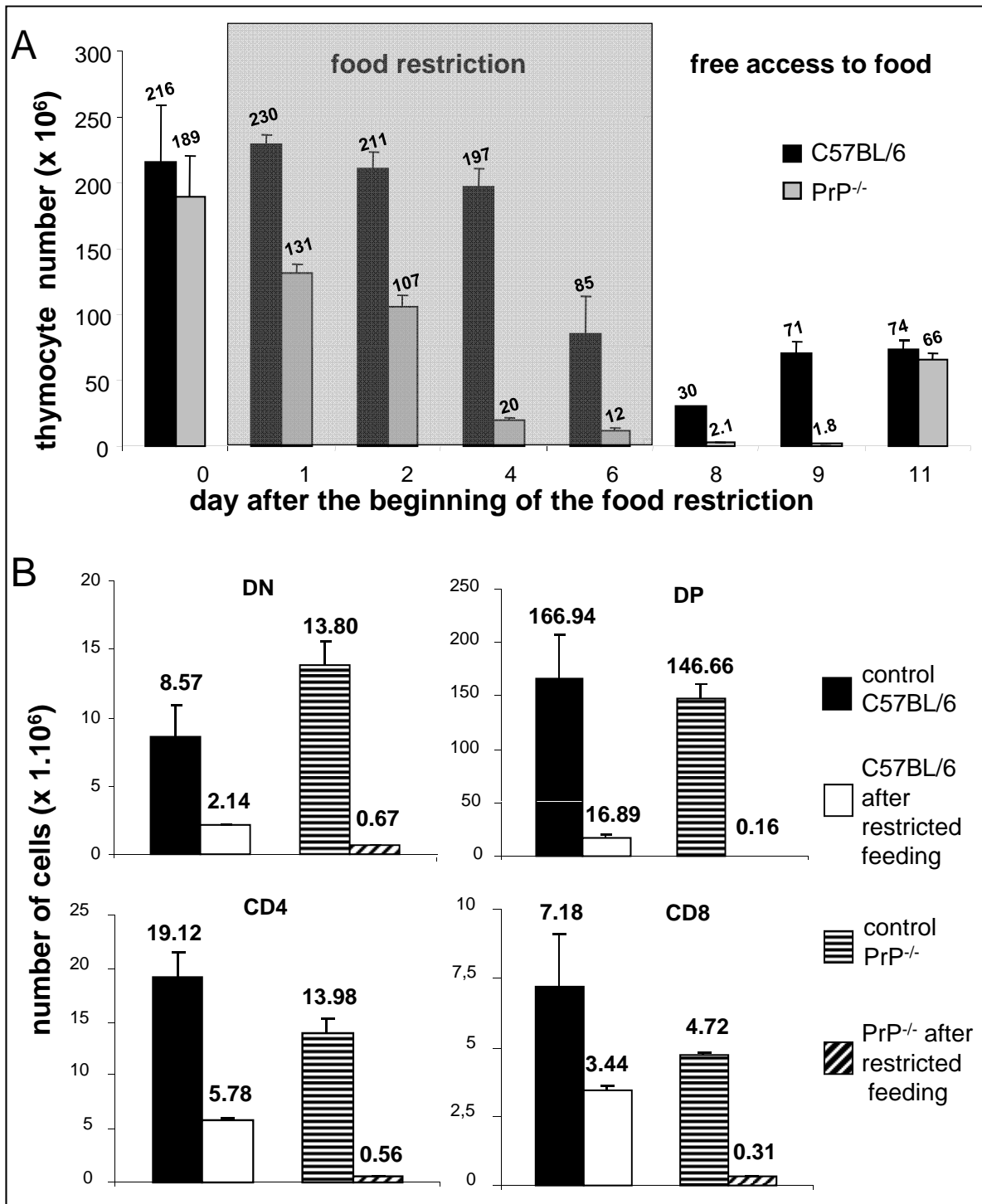
**Figure 1- Measurement of the intracellular reduced glutathione.** **A-**Thymocytes from C57BL/6 mice were labeled with anti-CD4, anti-CD8, anti-CD25 and anti-CD44 antibodies to define the different T cell subpopulations, and then incubated with monochlorobimane to evaluate their redox state, as described in “Materials and methods”. The data presented here are representative of four different experiments. **B-** Comparison of the level of GSH between C57BL/6 (black histograms) and PrP<sup>-/-</sup> (grey line) thymocytes prepared and analyzed as in A. The data shown here are representative of six different experiments.



**Figure 2- Effect of an oxidative stress on C57BL/6 and PrP-deficient thymocytes.** A- Control cells or cells that have been incubated with H<sub>2</sub>O<sub>2</sub> were stained with mCB and analyzed by flow cytometry as described in “Materials and methods”. The data presented here are representative of six different experiments on PrP<sup>+/+</sup> (C57BL/6) or PrP<sup>-/-</sup> mice. B- GRase activity in C57BL/6 and PrP<sup>-/-</sup> thymocytes: GRase activity was measured before (control) and after incubation of cells in the presence of H<sub>2</sub>O<sub>2</sub>. (\* p<0.05 ; n=3).



**Figure 3- Effect of a copper supplementation on the level of GSH.** Copper was given for five weeks in the drinking water of supplemented mice. Thymocytes were then recovered, submitted or not to H<sub>2</sub>O<sub>2</sub> and the level of mCB staining was determined as in Fig 2A.



**Figure 4- PrP<sup>-/-</sup> mice are more affected by a redox stress than C57BL/6 mice.** A: mice were allowed a free access to food and water for two hours per day for seven days. After this period, they recovered a free access to food and water. The cellularity of the thymus was evaluated every day. B: T cell number in each thymic subpopulation as determined by FACS analysis at day 8 after the initiation of the diet. Data is representative of n=4 for control mice and n=3 for the stressed mice.