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Impact of mouse pregnancy on thymic T lymphocyte subsets

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Abstract. It has been reported that fetal lymphoid progenitor cells are acquired during gestation and are able to develop in the maternal mouse thymus into functional T cells. Moreover, previous pregnancies increase the number of fetal cells in the mother. In the present study, we investigated whether mouse pregnancy induces changes in T lymphocyte subsets in the maternal thymus. We determined the T lymphocyte subsets in two allogeneic cross-breedings, namely CBA/J × BALB/c (normal) and CBA/J × DBA/2 (abortion prone), and investigated the effects of the age and parity of the female, as well as pregnancy outcome, on thymocyte populations. In addition, hormonal effects were evaluated in a syngeneic combination (CBA/J × CBA/J). We found that during pregnancy both hormonal and allogeneic stimuli induced a reduction in the $CD4^+CD8^+$ subset with an increase in the $CD4^+CD8^-$ population. Only young females of the normal combination exhibited an increase in the $CD4^-CD8^+$ population. All young mice showed an increase in $CD4^+CD25^+FoxP3^+$ T cells. Interestingly, the γδT thymus pool was increased in all females of the normal allogeneic pregnancy only, suggesting the participation of this pool in the observed beneficial effect of multiparity in this cross-breeding. Our results demonstrate that allogeneic pregnancies induce important variations in maternal thymocyte subpopulations depending on the age of the female and the male component of the cross-breeding.

Additional keywords: age, thymocytes.

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Introduction

The importance of several immunoregulatory factors that act in a coordinated manner at the maternal–fetal interface to successfully maintain an allogeneic pregnancy has been well described (Chaouat *et al.* 2010). However, the fact that fetal cells have been found in the circulation and persist in the periphery during the entire period of gestation as well as after delivery (Herzenberg *et al.* 1979; Liégois *et al.* 1981; Bianchi *et al.* 1996) suggests that the maternal immune system meets these foreign cells and is sensitised by them, acquiring specific tolerance (Tafuri *et al.* 1995; Aït-Azzouzene *et al.* 1998, 2001). Moreover, in an allogeneic model, Khosrotehrani *et al.* (2008) demonstrated that fetal lymphoid progenitor cells that were acquired during mouse pregnancy were able to develop in the maternal thymus into functional T cells, suggesting that these allogeneic cells would follow a similar selection course compared with maternal thymocytes.

It is well known that the thymus undergoes physiological changes associated with aging and pregnancy. The mouse thymus gains weight until the animals are approximately 5 weeks old (Hirokawa *et al.* 1994). Afterwards, the thymus begins its involution (Stutman and Good 1974), with loss of weight and cellularity (Aspinall 1997), while maintaining a significant level of activity (Hirokawa *et al.* 1982). Thymic involution also occurs during pregnancy (Persike 1940; Pepper 1961; Phuc *et al.* 1981; Kendall and Clarke 2000) and has been shown to be important for a normal pregnancy (Tibbetts *et al.* 1999). Progesterone and oestrogen have been proven to be part of this process (Chambers and Clarke 1979; Zoller and Kersh 2006). However, the allogeneic status of the conceptus has also been suggested to play a role in the induction or maintenance of thymus involution (Chaouat *et al.* 1982). Until now, there have been no studies into the possible relationship between the success or failure of a gestation and their effects on the thymus.

The development of T cells in the thymus starts when progenitor cells migrate to it from the bone marrow. For the αβ T cell lineage, cells double negative (DN) for CD4 and CD8 progress through a series of stages defined by CD44 and CD25 expression. Later, cells express a pre-T cell receptor (TCR) consisting of the rearranged β-chain in a complex with the pre-TCR α-chain, which induces the development to the double positive (DP) stage. These cells then rearrange the TCR α-chain and are selected based on the specificity of the αβ TCR for self-peptide major histocompatibility complex (MHC). Finally, cells undergo negative selection to eliminate reactive clones. Cells that successfully undergo these selection processes go on to

mature into $CD4^+$ or $CD8^+$ T cells, which are called single-positive (SP) thymocytes. The γδT cells originate in the thymus as a separate lineage (Chaplin 2003).

The CBA/J × DBA/2 cross-breeding was originally described as a high fetal-resorption model because of an altered immune response against the paternal antigens (Clark *et al.* 1980). In addition, recent studies have reported a reduced number of implantation sites (Prados *et al.* 2011) and suggest that the placentas in these mice present impaired angiogenesis and suffer from poor trophoblast invasion (Dixon *et al.* 2006; Litwin *et al.* 2010). Furthermore, we have observed that multiparity increases the grade of trophoblast invasion, even in the CBA/J × DBA/2 abortion-prone combination (Litwin *et al.* 2005, 2010). Even though multiparity has been associated with beneficial effects for ongoing gestation and the offspring, the effect of repetitive pregnancies on the thymus has not been investigated previously.

In the present study, we compared the effects of syngeneic and allogeneic pregnancies on the composition of thymocyte subpopulations and analysed a possible relationship of the pregnancy outcome in DBA/2-mated CBA/J females (abortion-prone cross-breeding) and BALB/c-mated CBA/J females (normal combination) with different age and parity status. To this end, we used term-pregnant mice because it has been demonstrated that the maximal passage of fetal cells occurs at this point of gestation (Vernochet *et al.* 2007).

Materials and methods

Animals

Two-month-old mice (CBA/J females and CBA/J, DBA/2 and BALB/c males) were purchased from Comisión Nacional de Energía Atómica (CNEA; Buenos Aires, Argentina). The CBA/J female mice were used as nulliparous virgin controls or were mated with either BALB/c male mice (CBA/J × BALB/c; normal $H\text{-}2^k \times H\text{-}2^d$ cross-breeding) or DBA/2 males (CBA/J × DBA/2; abortion-prone $H\text{-}2^k \times H\text{-}2^d$ cross-breeding) for the allogeneic combination or with CBA/J males (CBA/J × CBA/J; $H\text{-}2^k \times H\text{-}2^k$) for the syngeneic combination.

Pregnant female mice were divided into four groups: (1) primiparous young (PY): 3.0 ± 0.5 months old; (2) biparous young (BY): 4.0 ± 0.5 months old; (3) primiparous old (PO): 8.5 ± 0.5 months old; and (4) multiparous old (MO): 8.5 ± 0.5 months old with four pregnancies. The observation of vaginal plug denoted Day 0.5 of pregnancy. Nulliparous mice were divided in two groups of different based on age, namely those that were 3.0 ± 0.5 months old (NY) and those that were 8.5 ± 0.5 months old (NO). Mice were maintained on a 12-h light–dark cycle at constant temperature (20 ± 1°C). Mice from the BY group were killed 1 day after their second delivery. The rest of the mice were killed at 18.5 ± 1.0 days of pregnancy. The uterine horns were completely exteriorised to count the number of viable and dead or resorbing fetuses. An implantation site was counted as a conceptus whether it was viable or not. Many resorptions occur early in gestation and some resorbed conceptuses may not be apparent at 18.5 ± 1.0 days of pregnancy. Thus, we calculated this 'late resorption rate' as the number of resorptions/number of total implants × 100 at term pregnancy, as described by Chavez *et al.* (1987). We also scored the litter size and related its frequency to the total number of females studied. All experiments were performed according to the guidelines of the local institutional animal care committee (CICUAL) of the Faculty of Medicine, University of Buenos Aires.

Flow cytometry

The thymus was removed and placed in ice-cold RPMI 1640 medium (GIBCO BRL Invitrogen Corporation, Camarillo, CA, USA), and macerated through a fine wire mesh. The cell suspensions were then centrifuged at 200*g* for 10 min at 4°C. Erythrocytes were removed with buffer lysis (0.155 M NH_4Cl, 0.01 M $KHCO_3$, 0.001 M EDTA, pH 7.4) and washed with FCM buffer (phosphate-buffered saline (PBS), pH 7.4, with 1% bovine serum albumin (BSA) and 0.1% sodium azide). Cells were counted using a Neubauer chamber (Biotraza, Argentina).

Thymocytes (10^6 cells) were stained for 30 min at 4°C in the dark with allophycocyanin-cyanine 7 (APC-Cy7) rat anti-mouse CD4 clone GK1.5, phycoerythrin (PE) rat anti-mouse CD8a clone 53–6.7, fluorescein isothiocyanate (FITC) hamster anti-mouse γδ T-cell receptor clone GL3 (BD PharMingen; BD Biosciences, San Jose, CA, USA) and PE hamster anti-mouse αβ TCR clone H57–597 (Invitrogen Corporation). After washing with FCM buffer, cells were fixed with Fixation Buffer (1% paraformaldehyde) for 10 min at room temperature and then washed and preincubated with Permeabilisation Buffer (PBS, pH 7.4, 1% BSA, 0.1% sodium azide, 0.5% saponin) for 5 min before being stained with FITC Hamster anti-mouse CD3e clone 145–2C11 or PE-Cy7 hamster anti-mouse CD3e clone 145–2C11 (BD PharMingen) for 15 min in the dark at room temperature. Isotypic controls were used to rule out non-specific fluorescence.

Thymocytes (10^6 cells) were stained for T regulatory (T_{reg}) cells using a T_{reg} detection mouse kit (Miltenyi Biotec, Bergish Gladbach, Germany), which consists of APC monoclonal antibody (mAb) anti-FoxP3 clone 3G3, FITC mAb anti-CD4 clone GK1.5 and PE mAb anti-CD25 clone 7D4, according to the manufacturer's instructions.

Flow cytometry (FCM) analysis was performed using a FACSAria (Becton Dickinson, San Jose, CA, USA). A minimum of 10 000 events was collected for most analyses, with 30 000 events collected when $CD4^+CD25^+FoxP3^+$ and $TCR\gamma\delta^+$ T cells subsets were examined. The FCM data were analysed with WinMDI ver. 2.9 (Purdue University Cytometry Laboratories, Hansen Life Sciences Research Building, West Lafayette, IN, USA). In all cases, residual dead cells and cell aggregates were excluded by low angle and orthogonal light scatter. The strategy used to analyse the different subpopulations is given in Fig. S1, available as Supplementary Material for this paper. The $CD4^+CD8^+$, $CD4^+CD8^-$, $CD4^-CD8^+$, $CD3^+TCR\alpha\beta^+$ and $CD3^+TCR\gamma\delta^+$ subsets were analysed by gating on the $CD3^+$ population. The T_{reg} cells were studied by gating on $CD4^+$ subpopulation and scoring the CD25 and FoxP3 DP cells.

Statistics

The percentages obtained with the program WinMDI were transformed to proportions. Results are expressed as proportions

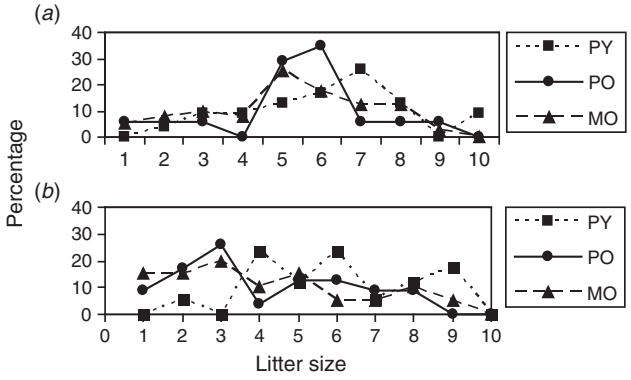

Fig. 1. Percentage of primiparous young (PY), primiparous old (PO) and multiparous old (MO) female mice with a given litter sizes on gestational Day 18.5 following (*a*) normal cross-breeding (CBA/J female mice × BALB/c male mice) and (*b*) abortion-prone cross-breeding (CBA/J × DBA/2).

for each single animal as a point and the mean ± s.d. for each group. Data were transformed using the arcsin function, with mean differences compared by ANOVA and the Newman–Keuls' multiple comparison test. Analyses were performed using GraphPad Prism 5 (Graph Pad Prism, La Jolla, CA, USA). $P < 0.05$ was considered significant.

Results

Effects of multiparity on late fetal resorption and the number of live fetuses

To compare the pregnancy outcome of both allogeneic cross-breedings, we evaluated the late resorption rate and litter size on gestational Day (GD) 18.5. Primiparous young females belonging to the normal cross-breeding showed a low resorption rate that increased slightly (but not statistically significant) in the older mice regardless of parity status (5 ± 3%, 10 ± 3% and 11 ± 3% in the PY ($n = 23$), PO ($n = 17$) and MO ($n = 27$) groups, respectively). In agreement with previous reports (Kiger *et al.* 1985; Clark *et al.* 1986; Gendron *et al.* 1990; Miranda *et al.* 1998; Blois *et al.* 2004), there was a higher number of resorbed fetus in primiparous young females belonging to the abortion-prone cross-breeding group and this was further increased with age (23 ± 2% and 33 ± 8% in the PY ($n = 17$) and PO ($n = 23$) groups, respectively; $P < 0.001$). Previous pregnancies did not improve this parameter (37 ± 7 in the MO group ($n = 20$); $P < 0.001$ vs PY).

The number of live fetuses in each group was determined and the results are shown in Fig. 1. Seventy per cent of primiparous females of the normal allogeneic mating combination had between five and eight litters, but this number was reduced in the primiparous old group: only approximately 64% had five to six fetuses. Noticeably, multiparity increased the number of live fetuses in old females to values similar to those observed in the young group: 67% of the females had between five and eight litters, of which 25% had seven to eight litters (Fig. 1*a*). Because of the number of live fetuses in females from the abortion-prone cross-breeding, a random pattern was observed across all groups studied (Fig. 1*b*). Only 35% of the multiparous females had between five and eight live fetuses. Consequently, in this cross-breeding there was no apparent beneficial effect of multiparity.

Pregnancy induces thymic involution in CBA/J females, enhancing the effect of age

To compare the extent of thymic involution among the different groups, we first analysed the total number of cells isolated from the thymus (Table 1). Cell number decreased with age, with an approximate 60% decrease between nulliparous female mice of 3 and 8.5 months of age; in addition a first pregnancy in young CBA/J females diminished thymus cellularity by approximately 90%, in agreement with the results reported by Phuc *et al.* (1981). Our data indicate that on GD 18.5, pregnancy had induced the same reduction in thymocyte number in all groups, regardless of age, cross-breeding and parity status. These results indicate that pregnancy enhances the effect of age on thymus cellularity and that repeated pregnancies do not affect this parameter. This effect remained constant 1 day after delivery, with $11.1 \pm 2.2 \times 10^6$ cells in the PY (CBA/J × CBA/J; $n = 4$) group and $12.2 \pm 3.1 \times 10^6$ cells in the BY (CBA/J × CBA/J; $n = 4$) group.

Pregnancy increases $CD4^+CD8^-$ subset but $CD4^-CD8^+$ T cells increase only in young normal pregnant females

We analysed $CD4^+CD8^+$ (DP), $CD4^+CD8^-$ and $CD4^-CD8^+$ (SP) lymphocyte subsets in the thymus. As shown in Fig. 2*a–c*, nulliparous old mice showed the same proportion of DP and both SP populations as the nulliparous young group. These results are in agreement with previously reported data, in which the thymic involution related to age did not cause variations in the proportion of T lymphocyte subsets (Aspinall 1997).

Interestingly, pregnancy reduced the proportion of DP T cells in all groups independent of age, parity status and cross-breeding (e.g. in young females, 0.79 ± 0.03, 0.44 ± 0.07 and 0.57 ± 0.04 for NY, CBA/J × BALB/c PY and CBA/J × DBA/2 PY, respectively; $P < 0.001$ vs PY for both cross-breedings; in old females, 0.79 ± 0.07, 0.53 ± 0.12 and 0.62 ± 0.06 for NO, CBA/J × BALB/c PO and CBA/J × DBA/2 PO, respectively; $P < 0.001$ and $P < 0.05$ vs NO, respectively). Multiparous females of both combinations also showed a diminished proportion of the DP subset with respect to the age control nulliparous group (0.63 ± 0.11 and 0.67 ± 0.07 for CBA/J × BALB/c MO and CBA/J × DBA/2 MO, respectively; both $P < 0.05$ vs NO) even though the normal combination showed an increase in the DP subset compared with primiparous old females of the same cross-breeding (CBA/J × BALB/c; $P < 0.05$). This effect was not noticeable in the abortion-prone cross-breeding (Fig. 2*a*).

Figure 2*b* shows that pregnancy increased the proportion of $CD4^+CD8^-$ in all groups regardless of age, parity status and cross-breeding (NY, 0.13 ± 0.02; NO, 0.12 ± 0.02; CBA/J × BALB/c PY, 0.30 ± 0.06; CBA/J × BALB/c PO, 0.25 ± 0.05; CBA/J × BALB/c MO, 0.23 ± 0.06; CBA/J × DBA/2 PY, 0.27 ± 0.03; CBA/J × DBA/2 PO, 0.23 ± 0.04; CBA/J × DBA/2 MO,

Table 1. Effects of age and pregnancy on thymic involution in CBA/J female mice

Results are the mean the absolute number of thymocytes per thymus ($\times 10^6$). *P*-values show the results of statistical analyses comparing the number of thymocytes between nulliparous females and nulliparous mice that were 3.0 ± 0.5 and 8.5 ± 0.5 months of age with pregnant mice that were the same age. The cross-breedings were of CBA/J female mice with BALB/c males (normal allogeneic cross-breeding) and of CBA/J female mice with DBA/2 male mice (abortion-prone allogeneic cross-breeding). ND, not detected; NY, nulliparous young; NO, nulliparous old; PY, primiparous young; PO, primiparous old; MO, multiparous old

	Nulliparous		CBA/J × BALB/c			CBA/J × DBA/2		
	NY	NO	PY	PO	MO	PY	PO	MO
n	9	7	9	7	9	5	8	9
Mean	140.80	91.71	13.09	12.37	10.17	21.50	19.50	20.81
s.d.	35.70	30.14	6.21	5.43	7.90	3.50	10.60	7.54
P-value		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

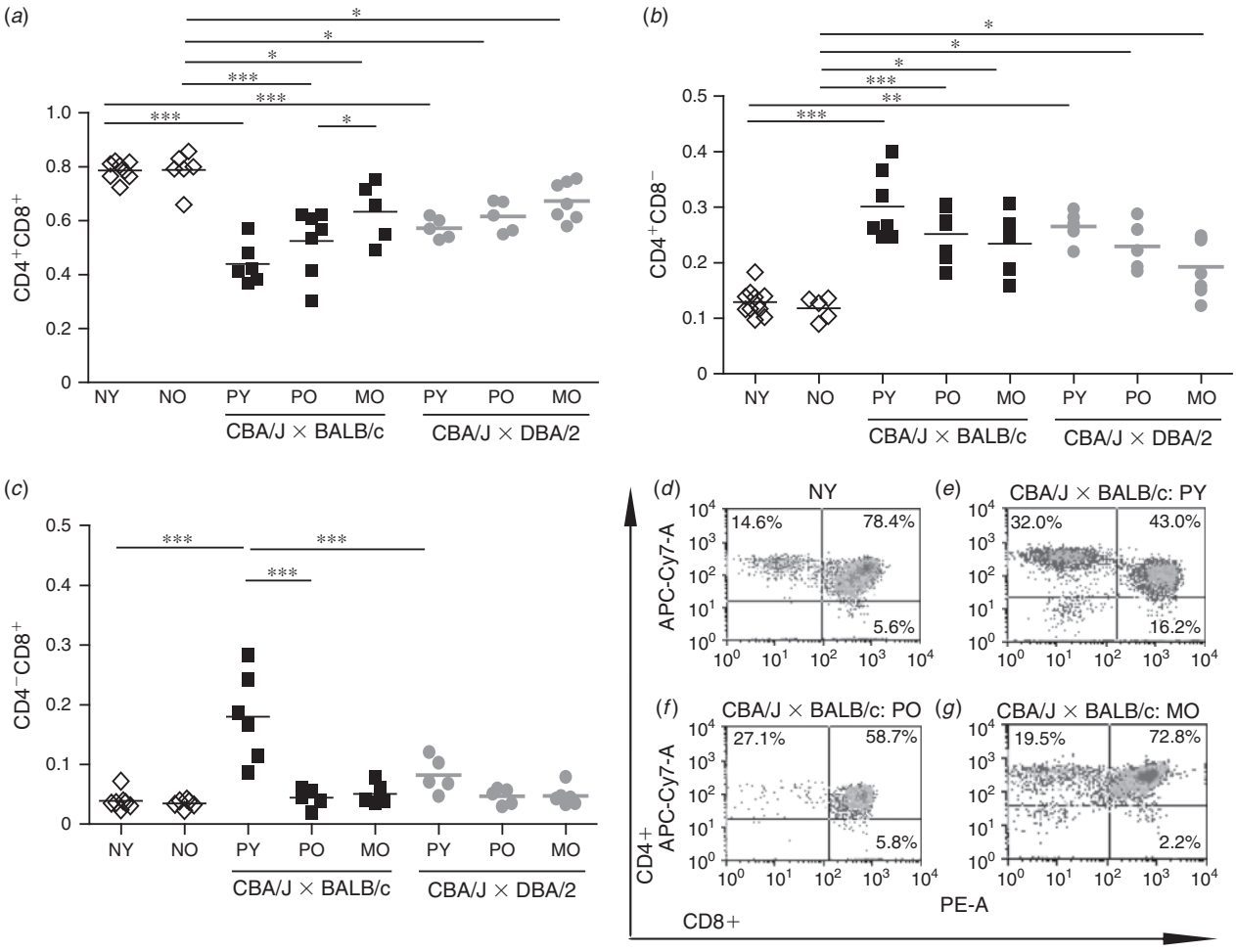

Fig. 2. Distribution of individual values for the proportion of (*a*) $CD4^+CD8^+$, (*b*) $CD4^+CD8^-$ and (*c*) $CD4^-CD8^+$ cells, as determined by flow cytometry. Thymocytes were gated according to forward and side scatter (FSC and SSC, respectively) and then gated according to $CD3^+$ expression. Percentages were transformed to proportions. Each point in the graph represents a single animal. Mean values are indicated by the bars. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (*d–g*) Representative density plots are shown for nulliparous young (NY) females (*d*), CBA/J × BALB/c primiparous young (PY) females (*e*), primiparous old (PO) females (*f*) and multiparous old (MO) females (*g*). (◇), NY; (■), normal cross-breeding (CBA/J females × BALB/c males); (●), abortion-prone crossbreeding (CBA/J × DBA/2).

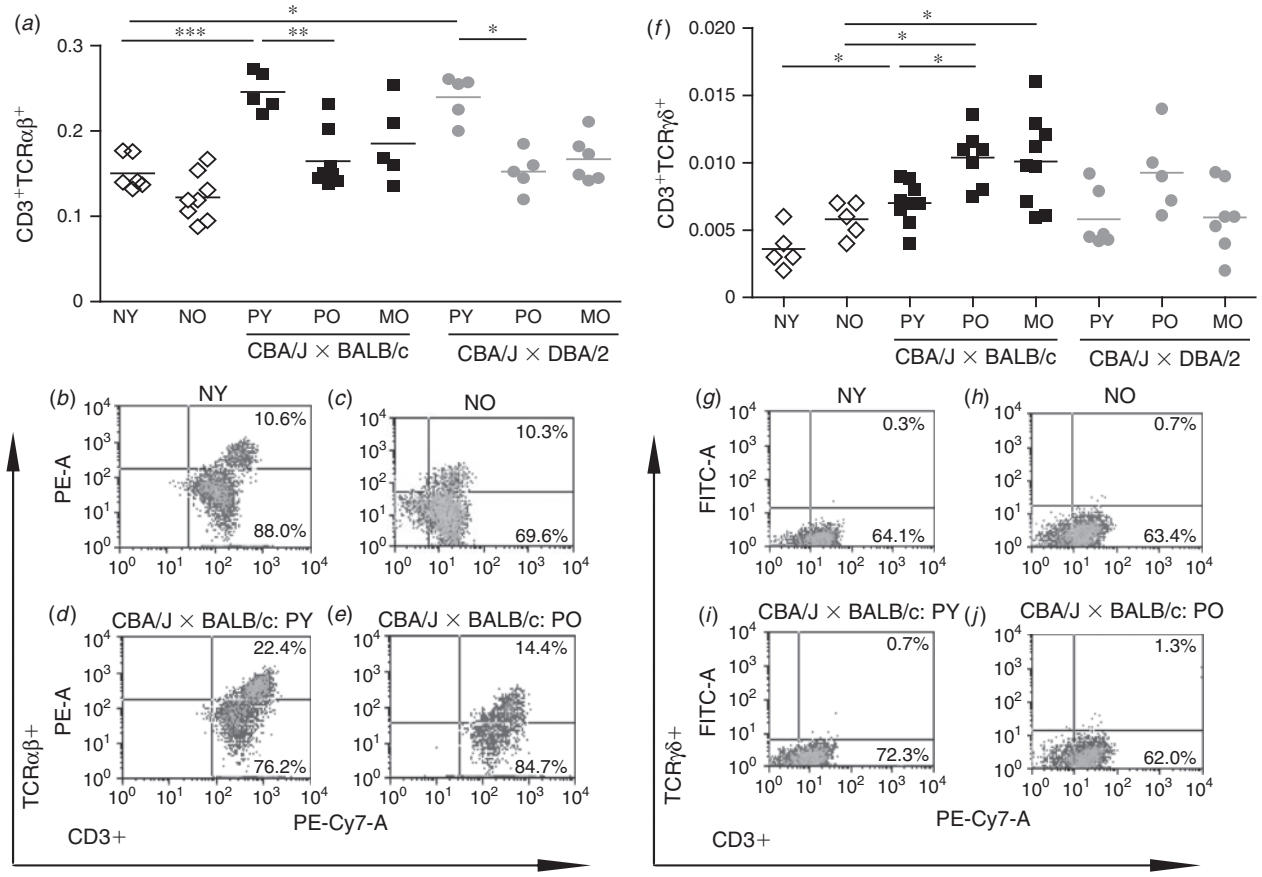

Fig. 3. Distribution of individual values of the proportion of (*a*) $CD3^+TCR\alpha\beta^+$ and (*f*) $CD3^+TCR\gamma\delta^+$ cells, as determined by flow cytometry. Thymocytes were gated according to forward and side scatter (FSC and SSC, respectively). Percentages were transformed to proportions. Each point in the graph represents a single animal. Mean values are indicated by the bars. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (*d–g*) Representative density plots are shown for $CD3^+TCR\alpha\beta^+$ in (*b*) nulliparous young (NY) and (*c*) nulliparous old (NO) females and (*d*) primiparous young (PY) and (*e*) primiparous old (PO) CBA/J × BALB/c females, as well as for $CD3^+TCR\gamma\delta^+$ cells in (*g*) NY, (*h*) NO and (*i*) PY and (*j*) PO CBA/J × BALB/c females. MO, multiparous old. (◇), NY; (■), normal cross-breeding (CBA/J females × BALB/c males); (●), abortion-prone crossbreeding (CBA/J × DBA/2).

0.19 ± 0.05; $P < 0.001$ for CBA/J × BALB/c PY vs NY; $P < 0.001$ for PO vs NO; $P < 0.05$ for MO vs NO; $P < 0.01$ for CBA/J × DBA/2 PY vs NY; $P < 0.05$ for PO and MO vs NO). However, as indicated in Fig. 2*c*, the $CD4^-CD8^+$ subset increased only in primiparous young females belonging to the normal combination (NY; 0.04 ± 0.02) compared with the age-matched control group (CBA/J × BALB/c PY; 0.18 ± 0.07; $P < 0.001$), the primiparous old of the same combination (PO; 0.04 ± 0.02; $P < 0.001$) and the primiparous young of the abortion-prone combination (CBA/J × DBA/2 PY; 0.08 ± 0.03; $P < 0.001$).

$TCR\alpha\beta^+$ T cells increase only in normal pregnant mice regardless of age and parity status

In the present study, we determined the proportion of thymocytes expressing TCRαβ (Fig. 3*a*) and TCRγδ (Fig. 3*f*). Young and old nulliparous groups showed the same proportion of $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ T cells, indicating no age-dependent effects in either case.

When the effect of a first pregnancy was analysed in young animals, an increase in the number of $TCR\alpha\beta^+$ T cells was noted in both cross-breedings compared with the age-matched control group (0.15 ± 0.02, 0.25 ± 0.02 and 0.24 ± 0.03 in the NY, CBA/J × BALB/c PY and CBA/J × DBA/2 PY groups, respectively; $P < 0.001$ and $P < 0.05$ vs NY, respectively). An increase in $TCR\gamma\delta^+$ cells was observed only in the normal combination (0.004 ± 0.001 and 0.007 ± 0.002 in the NY and CBA/J × BALB/c: PY groups, respectively; $P < 0.05$).

The effects of a first pregnancy in old females on the number of $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ T cells in the thymus differed. In the case of $TCR\alpha\beta^+$ T cells, no differences were observed between old females of the normal and abortion-prone cross-breedings compared with age-matched nulliparous females. However, primiparous old females had a lower number of $TCR\alpha\beta^+$ T

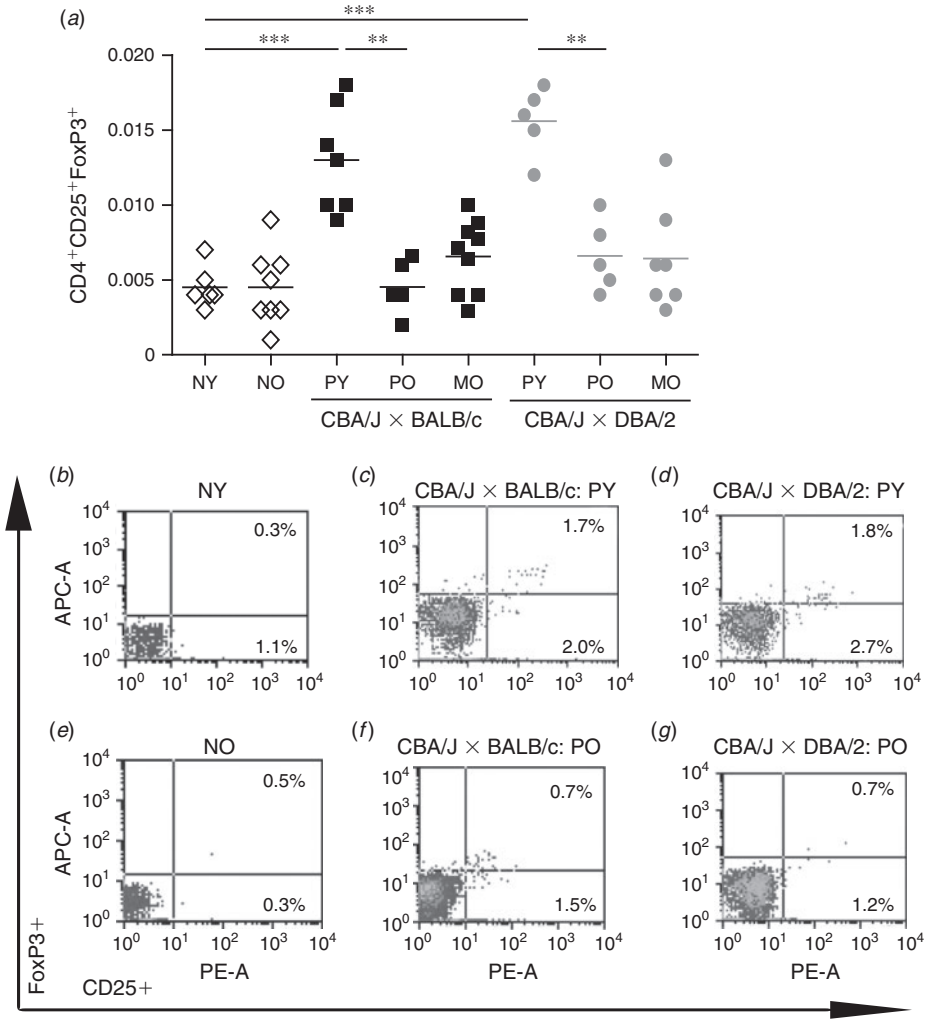

Fig. 4. Distribution of individual values of the proportion of $CD4^+CD25^+FoxP3^+$ cells, as determined by flow cytometry (*a*). Thymocytes were gated according to forward and side scatter (FSC and SSC, respectively) and then gated according to $CD4^+$ expression. Percentages were transformed to proportions. Each point in the graph represents a single animal. Mean values are indicated by the bars. $^{**}P < 0.01$, $^{***}P < 0.001$. (*b–g*) Representative density plots are shown for (*b*) nulliparous young (NY) and (*e*) nulliparous old (NO) females, (*c*) primiparous young (PY) and (*f*) primiparous old (PO) CBA/J × BALB/c females, and (*d*) PY and (*g*) PO CBA/J × DBA/2 females. MO, multiparous old. (◇), NY; (■), normal cross-breeding (CBA/J females × BALB/c males); (●), abortion-prone crossbreeding (CBA/J × DBA/2).

cells than the primiparous young females (0.16 ± 0.03 and 0.15 ± 0.02 in CBA/J × BALB/c PO and CBA/J × DBA/2 PO groups, respectively; $P < 0.01$ and $P < 0.05$ vs PY, respectively). The number of $TCR\gamma\delta^+$ T cells in old females of the normal cross-breeding increased (0.006 ± 0.001 and 0.010 ± 0.002 in the NO and CBA/J × BALB/c PO groups, respectively; $P < 0.05$). In this case, normal primiparous old females had a higher number of $TCR\gamma\delta^+$ T cells than primiparous young females ($P < 0.05$).

There were no significant differences in $TCR\alpha\beta^+$ or $TCR\gamma\delta^+$ T cells in multiparous females with respect to age-matched primiparous females, indicating that multiparity status *per se* did not modify these parameters.

Increased $CD4^+CD25^+FoxP3^+$ cells only in young pregnant mice from the normal and abortion-prone cross-breeding

In the present study we analysed the $CD4^+CD25^+FoxP3^+$ population generated in the thymus, taking into account that these three markers phenotypically identify T_{reg} cells (Fontenot *et al.* 2003; Hori *et al.* 2003; Khattri *et al.* 2003). Figure 4 shows that non-pregnant CBA/J females that were 3 and 8.5 months of

Table 2. Effect of a second pregnancy on thymocyte subsets in CBA/J females

Results are the mean ± s.d. proportion of the marker. The cross-breedings were of CBA/J female mice with either CBA/J male mice (syngeneic) or BALB/c males (normal allogeneic cross-breeding) and of CBA/J female mice with DBA/2 male mice (abortion-prone allogeneic cross-breeding). ND, not detected; NY, nulliparous young; PY, primiparous young; BY, biparous young. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs NY group, $\dagger P < 0.05$; $\dagger\dagger P < 0.01$ vs PY of CBA/J × CBA/J

Crossbreeding		CBA/J × CBA/J		CBA/J × BALB/c		CBA/J × DBA/2	
Groups	NY	PY	BY	PY	BY	PY	BY
$CD4^+CD8^+$	0.79 ± 0.03	0.62 ± 0.12	0.64 ± 0.11	0.44 ± 0.07	0.49 ± 0.09	0.57 ± 0.04	0.64 ± 0.09
$CD4^+CD8^-$	0.13 ± 0.02	0.21 ± 0.09	0.23 ± 0.07	0.30 ± 0.06	0.32 ± 0.08	0.27 ± 0.03	0.30 ± 0.07
$CD4^-CD8^+$ s.d.	0.04 ± 0.02	0.04 ± 0.03	0.07 ± 0.03	0.18 ± 0.07	0.15 ± 0.05	0.08 ± 0.03	0.06 ± 0.04
$CD4^+CD25^+FoxP3^+$	0.005 ± 0.001	0.010 ± 0.002	ND	0.013 ± 0.004	ND	0.016 ± 0.002	ND
$CD3^+TCR\gamma\delta^+$	0.004 ± 0.001	0.005 ± 0.002	0.006 ± 0.003	0.007 ± 0.002	0.008 ± 0.003	0.006 ± 0.002	0.007 ± 0.003
n	6	4	4	5	4	5	4

age had a similar proportion of $CD4^+CD25^+FoxP3^+$ cells (0.005 ± 0.001 and 0.005 ± 0.002, respectively) and that pregnancy in both normal (CBA/J × BALB/c PY) and abortion-prone (CBA/J × DBA/2 PY) young mice increased the expression of these cells (0.013 ± 0.004 and 0.016 ± 0.002, respectively; $P < 0.001$ vs non-pregnant mice for both cross-breedings). This effect was not observed in old pregnant mice (either primiparous or multiparous) of either cross-breeding compared with nulliparous old mice.

Allogeneic pregnancy modifies the proportion of $CD4^+CD8^+$, $CD4^+CD8^-$, $CD4^-CD8^+$ and $TCR\gamma\delta^+$ but not $CD4^+CD25^+FoxP3^+$ T cells

To evaluate the hormone influence in the variations observed in thymic T lymphocytes subsets in pregnant mice, we studied the proportion of DP $CD4^+CD8^+$, SP $CD4^+CD8^-$, SP $CD4^-CD8^+$, $CD4^+CD25^+FoxP3^+$ and $TCR\gamma\delta^+$ T lymphocytes in the thymus of young females of the CBA/J × CBA/J combination. The results are given in Table 2. A similar diminished proportion of DP cells was seen in the syngeneic combination and in the abortion-prone cross-breeding, which was lower than that in nulliparous females ($P < 0.001$; Table 2). The normal allogeneic combination resulted in the greatest reduction in DP cells (Table 2).

In the case of $CD4^+CD8^-$ cells, both the syngeneic and the abortion-prone gestation increased their proportion in the thymus with respect to nulliparous young females (Table 2). However, the major effect was observed in normal young mice ($P < 0.001$ for NY vs PY CBA/J × BALB/c; $P < 0.05$ for PY CBA/J × CBA/J vs PY CBA/J × BALB/c). These data indicate that even though the proportion of DP T cells and $CD4^+CD8^-$ T cells is influenced by the hormonal environment in pregnancy, it is also affected by allogeneic gestation. As indicated in Table 2, the proportion of $CD4^-CD8^+$ T and $TCR\gamma\delta^+$ T cells increased only in the normal cross-breeding and no effect was observed for either syngeneic gestation or the abortion-prone combination.

The proportion of $CD4^+CD25^+FoxP3^+$ T cells was increased by syngeneic pregnancy to a similar percentage as observed in the normal cross-breeding (Table 2). The abortion-prone cross-breeding resulted in a higher proportion of $CD4^+CD25^+FoxP3^+$ T cells compared with the syngeneic combination ($P < 0.01$ for PY CBA/J × CBA/J vs PY CBA/J × DBA/2), but it did not differ significantly compared with the normal combination. These results indicate that the variations in the proportion of $CD4^+CD25^+FoxP3^+$ T cells in the thymus of young pregnant females respond exclusively to a hormonal effect and not to allogeneic gestation.

Parturition diminishes the $CD4^+CD25^+FoxP3^+$ pool in the thymus

To investigate variations in T cell subsets due to sensitisation by parturition in young mice, we further examined the proportion of the same T cell subsets in the thymus of females 1 day after their second delivery (BY groups). We used the CBA/J × CBA/J, CBA/J × BALB/c and the CBA/J × DBA/2 combinations and compared the results obtained for the BY groups with those obtained in a first pregnancy (PY groups). The results are given in Table 2. For each mating combination, the BY groups showed similar values for T subsets analysed compared with their respective PY groups, except for the $CD4^+CD25^+FoxP3^+$ pool, suggesting that the modifications in the T cell subsets in the thymus are generated during gestation and are not a consequence of sensitisation by parturition due to blood exchange between mother and fetus. Interestingly, $CD4^+CD25^+FoxP3^+$ cells were not detected in the thymus of any BY groups.

Discussion

In the present study we investigated the impact of gestation on thymic T lymphocyte subsets and pregnancy outcome in two $H\text{-}2^k \times H\text{-}2^d$ allogeneic mouse cross-breedings, namely CBA/J females mated with BALB/c (normal combination) or DBA/2 (abortion-prone cross-breeding) male mice. Moreover, the effects of the age and parity status of the dam were evaluated. We also included a CBA/J × CBA/J mating to investigate differences between hormone and immunological events.

We first observed that the atrophy induced by pregnancy was stronger than that induced by age. Furthermore, all term-pregnant females and those killed 1 day after delivery had a similar number of thymocytes, indicating that thymus involution was not affected by the cross-breeding, parity status or

delivery. Therefore, we next compared the proportions of LT subpopulations among the groups.

Different effects of pregnancy on the percentage of SP thymocyte subsets have been reported in the literature (Clarke and Miller 1991; Brunelli *et al.* 1992; Zoller *et al.* 2007). In the present study, we found a strong reduction in the DP subset with a concomitant increase in SP $CD4^+$ cells in both allogeneic cross-breedings compared with age-matched nulliparous female groups. Interestingly, only young females from the normal CBA/J × BALB/c mating combination exhibited a significant increase in the SP $CD8^+$ population and this effect was not seen in any of the old female mice. Conversely, we found that both normal and abortion-prone young pregnant females exhibited an increase in αβT cells compared with the age-matched control. Our experiments using the syngeneic combination suggest that variations in DP and SP $CD4^+CD8^-$ T cells depend on both hormonal and allopregnancy stimuli. The proportion of $CD4^-CD8^+$ T cells increased only in the normal cross-breeding, and not in either the syngeneic gestation or in the abortion-prone combination, indicating in this case the requirement of a normal allogeneic pregnancy.

During mouse pregnancy, an increase in $CD4^+CD25^+$ cells has been reported in the spleen, lymph nodes, blood and uterus (Aluvihare *et al.* 2004). As is the case in humans, many reports suggest that T_{reg} cells are involved in the maintenance of tolerance during a successful mouse pregnancy (Aluvihare *et al.* 2004; Zenclussen *et al.* 2005; Kallikourdis *et al.* 2007). In the thymus, Zoller *et al.* (2007) found an increase in the percentage of $CD4^+CD25^+$ cells in C57BL/6 mice at term for both syngeneic and allogeneic matings. Considering the current consensus, in the present work we used the coexpression of CD4, CD25 and FoxP3 markers to phenotypically identify T_{reg} cells. Our results showed that young pregnant females belonging to the normal CBA/J × BALB/c cross-breeding exhibited an increased proportion of $CD4^+CD25^+FoxP3^+$ cells in the thymus. Interestingly, we report for the first time that young pregnant female mice of the CBA/J × DBA/2 abortion-prone cross-breeding exhibited the same high level of $CD4^+CD25^+FoxP3^+$ cells as the normal combination at the end of gestation. These data indicate that there are no quantitative differences in the thymus pool of T_{reg} cells between the two cross-breedings. Conversely, old pregnant mice of both combinations did not show an increase in the thymic proportion of $CD4^+CD25^+FoxP3^+$ cells, with similar values to those seen in age-matched nulliparous females. It has been reported that decidual $CD4^+CD25^{bright}$ T cells from normal pregnancies have increased expression of surface cytotoxic T-lymphocyte antigen 4 (CTLA-4), indicating that these cells have already been stimulated on the TCR, perhaps by fetal antigens (Sasaki *et al.* 2004). Furthermore, $CD4^+CD25^+$ cells expressing $CCR5^+$, which is associated with a highly suppressive phenotype, have been found in the gravid uterus, especially in allogeneic pregnancies, suggesting that these cells are activated by fetal antigens in the periphery (Kallikourdis *et al.* 2007). Until now, it has not been possible to distinguish whether these T_{reg} cells where generated in the thymus or in the periphery. Curiously, in the present study we did not detect $CD4^+CD25^+FoxP3^+$ cells in the thymus of females 1 day after their second delivery. One possible explanation for this may be that these cells could have migrated to the periphery as a consequence of sensitisation by parturition. No variations were observed in the other T lymphocyte subsets.

Our results also showed upregulated expression of γδT cells in the mouse thymus due to pregnancy. Interestingly, this effect was observed only in the normal cross-breeding independent of age and the number of previous pregnancies. These data could explain the marked increase in pregnant mouse endometrium previously reported (Meeusen *et al.* 1993). Some evidence supports the idea that these cells may exert regulatory functions in pregnancy and cooperate in fetal tolerance. Szekeres-Bartho *et al.* (1999) reported that the number of γδT cells in the mouse uterus is higher in allogeneic than in syngeneic pregnancies. In addition, Arck *et al.* (1997, 1999) observed that two populations of γδT cells arise in mouse deciduas: an early T helper (TH) 1 population present during the time of implantation that is abortogenic if it persists and a TH2/3 cell subset that appears later during pregnancy with anti-inflammatory and pregnancy-protective properties. Moreover, in healthy pregnant women, an accumulation of $V\delta1^+$ circulating cells has been reported in contrast with women with recurrent abortions, in whom $V\delta2^+$ circulating cells dominated (Gilpin *et al.* 1998; Shi *et al.* 2000; Chafetz *et al.* 2007). A bias towards circulating $V\delta1^+\gamma\delta T$ cells seems to be required for a successful normal pregnancy (Szekeres-Bartho *et al.* 2001).

In contrast with $CD4^+CD25^+FoxP3^+$ cells, we found that the increase in γδT cells in the thymus persists at least until pregnant mice were 9 months old. These two types of cells belong to different lineages, so it is conceivable that an old thymus can generate one type of cell and not the other. Because this effect was observed only in the normal combination, and taking into account the already reported protective properties of γδT cells, we suggest that these cells may contribute to the maintenance of gestation at an advanced age when the ability of the thymus to generate T_{reg} cells is reduced. We observed that although the effects of pregnancy on $CD4^+CD25^+FoxP3^+$ T cells may depend exclusively on the hormonal environment related to pregnancy, the effects of pregnancy on γδT cells may depend solely on the allogeneic stimulus.

In the present study, multiparous females of the normal cross-breeding showed a higher proportion of the DP subset compared with age-matched primiparous females. However, the remaining markers did not vary between the cross-breedings, indicating that multiparity status had no effect on the thymus, at least during the current gestation at term. It has been demonstrated that virgin female mice of certain strains reject male skin grafts due to the H-Y antigen. However, after many syngeneic pregnancies, when mice were between 6 and 9 months of age, most of the females became tolerant of such grafts (Billingham *et al.* 1965; Simpson *et al.* 1981; James *et al.* 2003) and thymus-dependent cells from multiparous tolerant donors were able to transfer adoptively this pregnancy-induced tolerance (Smith and Powell 1977). Until now, the nature of the maternal T cells responsible for this effect has not been described. Taking into account the age of the mice in the previous studies and the results of the present study, it may be that thymus γδT cells, and not $CD4^+CD25^+FoxP3^+$ cells, are

responsible, in part, for the pregnancy-induced tolerance to H-Y in aged mice.

A possible role for placental cells in the immunoregulation of the functional activity of thymocytes was first reported by Savion and Toder (1995). Later, Vallejo *et al.* (2009) showed that pregnancy-associated plasma protein A (PAPP-A), a metalloproteinase secreted by mouse placenta that controls the tissue availability of insulin-like growth factor (IGF), is involved in thymic atrophy and decreases T cell activation during pregnancy. In addition, PAPP-A, a disintegrin and metalloproteinase (ADAM) 12 and placental protein 13 (PP13) are thought to be involved in normal implantation and placental development. Recently, Sahraravand *et al.* (2011) observed a correlation between the size of the human placenta and the secretion of PAPP-A, ADAM12 and PP13. Thus, differences in the secretion or functionality of these proteins may be related to the different effects on the thymus of pregnant mice described in the present study.

Our results confirmed that old CBA/J females (of any parity status) mated with DBA/2 males have a higher abortion rate than young mice. However, the number of live fetuses did not vary among the groups. Instead, the normal mating combination always showed a low abortion rate regardless of the age and parity status. Interestingly, only multiparous females mated with BALB/c males exhibited a significantly increased litter size. Because primiparous and multiparous old females of the normal combination showed a similar high proportion of γδT cells, the beneficial effect of multiparity may not to be related solely to this effect. Moreover, normal primiparous young females had a similar proportion of $CD4^+CD25^+FoxP3^+$ lymphocytes and a slightly higher proportion of γδT cells than that observed in the abortion-prone combination, but these two groups clearly had different pregnancy outcomes, Hence, the results suggest that peripheral tolerance mechanisms play a crucial role in the success of pregnancy.

In conclusion, our results show that pregnancy in allogeneic mating combinations induces important quantitative variations in maternal thymocyte subpopulations according to the age of the female and the male component of the cross-breeding (BALB/c or DBA/2). Having observed this effect, we are currently studying the functional behaviour of the thymocytes from the different groups investigated herein.

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