Distinct Mechanisms of Neonatal Tolerance Induced by Dendritic Cells and Thymic B Cells

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Summary

To assess the role of different types of antigen-presenting cells (APC) in the induction of tolerance, we isolated B cells, macrophages, and dendritic cells from thymus and spleen, and injected these into neonatal BALB/c mice across an Mls-1 antigenic barrier. One week after injection of APC from Mls-1-incompatible mice or from control syngeneic mice, we measured the number of thymic, Mls-1^a-reactive, $V_\beta 6^+$ T cells and the capacity of thymocytes to induce a graft-vs.-host (GVH) reaction in popliteal lymph nodes of Mls-1^a mice. Injection of thymic but not spleen B cells deleted thymic, Mls-1^a-reactive $V_\beta 6^+$ T cells and induced tolerance in the GVH assay. The thymic B cells were primarily of the $CD5^+$ type, and fluorescence-activated cell sorter–purified $CD5^+$ thymic B cells were active. Injection of dendritic cells from spleen or thymus also induced tolerance, but the $V_\beta 6$ cells were anergized rather than deleted. Macrophages from thymus did not induce tolerance. Dendritic cells and thymic B cells were also effective in inducing tolerance even when injected into Mls^-, major histocompatability complex–incompatible, $I\text{-}E^-$ mice, but only thymic B cells depleted $V_\beta 6$-expressing T cells. Therefore, different types of bone marrow–derived APC have different capacities for inducing tolerance, and the active cell types (dendritic cells and $CD5^+$ thymic B cells) can act by distinct mechanisms.

One of the important functions of the thymus gland is to inactivate lymphocytes that would react against self-constituents (1). Tolerance can develop in at least two ways: clonal deletion (2–5) and clonal anergy (6–8). The latter refers to a phenomenon whereby self-reactive T cells are present but are unable to make an effective response to antigen. T cells expressing both α/β and γ/δ types of TCR can be tolerized in the thymus (9–13). There is evidence that thymic APC derived from the bone marrow are somehow important in bringing about tolerance (12, 14), one such model being the induction of tolerance to an antigen called Mls (15). Mls is an attractive model since specific TCR V_β genes encode reactivity to specific alleles of Mls, enabling one to enumerate Mls-reactive T cells (3, 5).

We have noted that the mouse thymus contains a small number of B cells, the majority of which express the CD5 molecules (16). B cells are known to be the major type of APC that express Mls (17–19). We have now prepared highly enriched populations of B cells from thymus and spleen and injected these into the thymi of mice at the neonatal period, when mice are most susceptible to the induction of tolerance (20–22). We find that B cells from thymus, including those bearing CD5, and activated B blasts, but not resting spleen B cells, can induce tolerance by deleting Mls-reactive T cells. When other APC are tested, dendritic cells but not macrophages also can tolerize to Mls, but the dendritic cells do so by inducing clonal anergy rather than deletion.

Materials and Methods

Mice. Mice with different Mls and MHC antigens were obtained from Japan SLC; Shizuoka and Clea Japan; Osaka, Japan. The strains were DBA/2,H-2^d, Mls-1^a; BALB/c, H-2^d, Mls-1^b; BALB/c × DBA/2 F_1 (CxD2 F_1), H-2^d, Mls-$1^{b/a}$; C57BL/6, H-2^b, Mls-1^b, C57BL/6 × DBA/2 (B6xD2 F_1) H-$2^{b/d}$, Mls-$1^{b/a}$; BALB/c × C57BL/6 (CxB6 F_1), H-$2^{d/b}$, Mls-1^b; C3H/He, H-2^k, Mls-1^b; B6.H-2^k, H-2^k, Mls-1^b; and AKR/J, H-2^k, Mls-1^a.

APC. Dendritic cells and thymic B cells were prepared as described elsewhere (16, 23). These APC were >98% Ig^- and >95% Ig^+, respectively, and lacked detectable macrophages stained with F4/80 mAb. Briefly, dendritic cells were prepared from collagenase-digested organs that were centrifuged in dense BSA columns to provide low-density adherent populations. After over-

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night culture, dendritic cells became nonadherent, while macrophages remained adherent but could be dislodged after 30 min of culture in PBS with 1 mM EDTA at 37°C. In some experiments, dendritic cells were further purified by treatment with anti-B cell mAb (RA3-3A1/6.1;TIB146 or J11d.2;TIB183) plus rabbit complement. Depletion of B cells was monitored by the absence of mitogen responses to LPS. Positive selection of dendritic cells was performed by sorting as $N418^+$ (24) cells using a FACStar Plus® (Becton Dickinson & Co., Mountain View, CA) according to method described (25). Thymic B cells were prepared from teased thymi that were centrifuged in dense Percoll columns to provide a low-density population. The low-density fraction from the teased thymi had only trace levels of dendritic cells, and was depleted of T cells by treatment with a mixture of anti-Thy-1.2 (HO-13-4), CD4 (GK1.5), and CD8 (HO-2.2 and 3.155) mAb and complement. Anti-CD5 mAb (C3PO; American Type Culture Collection, Rockville, MD) was used to deplete $CD5^+$ B cells (~75% of the total) in the thymic B cell population, while biotinylated anti-CD5 and PE-avidin (Becton Dickinson & Co.) were used to sort the $CD5^+$ B cells on a FACStar instrument (Becton Dickinson & Co.). Splenic B cells were Sephadex G-10 nonadherent cells depleted of T cells by anti-Thy-1.2 mAb and complement, followed by Percoll density gradient centrifugation to obtain high-density resting B cells. Splenic B cells cultured for 24 h with goat $F(ab')_2$ anti-mouse IgM Ab (Tago Inc., Burlingame, CA) plus murine IL-4 served as B blasts.

Neonatal Tolerance Regimen to Mls, and Frequency of Mls-reactive T Cells. BALB/c Mls-1^b newborn mice within 24 h of birth were anesthetized with diethyl ether and fixed onto a board with rubber bands. The mediastinum was opened and 0.5 μl of an APC cell suspension (5×10^5) was injected directly into each lobe of the thymus using a 5-μl microsyringe. The APC were from incompatible Mls-1^a or from syngeneic Mls-1^b mice. The chest was then closed by suturing the overlaying skin. 1 wk later, the thymus or pooled peripheral lymph nodes (axillary, inguinal, brachial, mesenteric) were removed and tested for Mls reactivity (below) or for the frequency of cells expressing different TCR V_β antigens by FACScan and Consort 30 program (Becton Dickinson & Co.). The anti-$V_\beta 6$ and anti-$V_\beta 8$ mAbs were rat 44-22-1 and mouse F23.1, respectively, and these were visualized with FITC mouse anti-rat Ig (Boehringer Mannheim Biochemicals, Indianapolis, IN) and goat anti-mouse Ig (Cappel Laboratories, Malvern, PA). As will be evident in the results, anti-$V_\beta 6$ cells were tolerized while most $V_\beta 8$ cells were not, even though some $V_\beta 8.1$ cells are Mls-1^a reactive (3).

Responses to Mls In Vivo. Different APC, including $CD5^+$ B cells enriched by sorting thymic B cells on a FACStar after staining with biotinylated anti-CD5 plus PE-avidin mAb (26), were injected intrathymically into BALB/c neonatal mice. 1 wk later, reactivity of these tolerized cells to Mls-1^a in vivo was assessed by injecting 2×10^7 thymocytes in 25 μl into the hind footpad of adult mice that were disparate at Mls-1^a or MHC loci or at both loci. Lymph node swelling as a measure of a local graft-vs.-host reaction (GVHR)[1] was performed 7 d later (27). Percent responsiveness was calculated according to the formula: 100× [(lymph node weight in experimental mouse − the mean weight in negative control)/(the mean weight in positive control − the mean weight in negative control)]. The positive controls were given thymocytes from BALB/c mice and the negative controls were given thymocytes from age-matched syngeneic CxD2 mice. This assay may measure tolerance to Mls^a as well as tolerance to other histocompatibility differences between BALB/c and DBA/2.

Responses to Mls In Vitro. Expression of Mla-1^a by APC was evaluated with a MLR in vitro. $CD4^+$ T cells from BALB/c or CxB6 mice or from control CxD2 mice were prepared by passing a mixture of spleen and lymph node suspensions over nylon wool columns and then depleting $CD8^+$ cells with anti-CD8 mAb plus rabbit complement. The stimulators were from Mls-1^a CxD2 or B6xD2 mice or MHC-incompatible B6.H-2^k mice. The APC were treated with 40 μg/ml of mitomycin C for 30 min at 37°C before responder T cells, 3×10^5 per flat-bottomed microtest well, were added. The culture medium was 0.2 ml of RPMI 1640 containing 10% FCS, 50 μM 2-ME, and 100 μg/ml gentamicin. DNA synthesis was determined as [^{3}H]thymidine incorporation (1 μCi/ml, 6 Ci/mM) at 72–84 h. Results are the mean of triplicate cultures. The frequency of responding T cells expressing $V_\beta 6$ or $V_\beta 8$ was assessed at day 3 using FACS analyses (above) of the small and large cell fractions, as distinguished by forward light scattering and as illustrated in Results.

Responsiveness to immobilized anti-$V_\beta 6$, anti-$V_\beta 8$ and anti-CD3 mAb was measured by proliferation of B cell–depleted lymph node cells (axillary, brachial, and mesenteric nodes) obtained from mice that were injected intrathymically with APC 9 d before. The anti-$V_\beta 6$, anti-$V_\beta 8$, and anti-CD3 mAbs in culture supernatant were fixed onto tissue culture plates by 50 μg/ml of goat anti–rat IgG, anti-mouse IgG, and anti–hamster IgG, respectively. Results are the means of duplicate cultures.

Results

Presentation of Mls Antigens by Thymic B Cells and Other APC. We first compared the capacity of $CD5^+$ B cells and dendritic cells from the thymus to present Mls alloantigens to T cells in vitro, since this had not been studied previously. $CD4^+$ T cells were used as responders, since the bulk of the

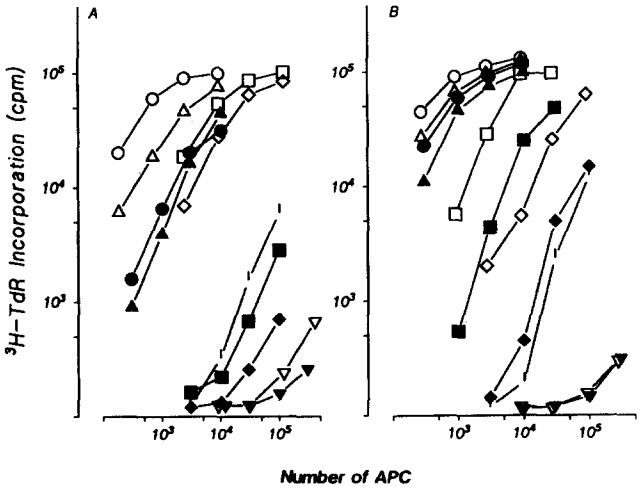

Figure 1. Stimulatory activity of dendritic cells and thymic B cells during the in vitro response to Mls-1^a. The T cells were $CD4^+$ cells from BALB/c mice (*A*). The APC were dendritic cells from thymus (○, ●) and spleen (△, ▲), bulk thymic B cells (◇, ◆), thymic B cells depleted of $CD5^+$ cells (▮), B blasts (□, ■), and resting splenic B cells (▽, ▼) from incompatible CxD2 F_1 (H-2^d, Mls-1^a) (*open symbols*) and BALB/c (H-2^d, Mls-1^b) (*closed symbols*). Responses of $CD4^+$ T cells from MHC-incompatible C57BL/6 mice (H-2^b, MLS-1^a) served as positive controls for the BALB/c APC (*B*).

[1] *Abbreviation used in this paper:* GVHR, graft-vs.-host reaction.

Table 1. *Stimulatory Activity of Purified Dendritic Cells to Mls-disparate T Cells*

Exp.	T cells from:	Dendritic cells			T cell proliferation			
		Strain	Organ	Purification	3 × 10⁴*	10⁴	3 × 10³	10³
						× 10⁻³ cpm		
1	Syngeneic (BALB/c)	BALB/c	Spleen	Cultured	11.5	9.1	4.9	1.8
				Cultured FcR⁻	10.3	9.6	4.5	1.2
				Cultured 146⁻	12.4	10.7	4.1	0.8
				Cultured J11d⁻	9.8	7.9	2.9	0.4
	Mls-incompatible (BALB/c)	CxD2	Spleen	Cultured	84.4	51.6	32.5	13.2
				Cultured FcR⁻	85.3	53.2	31.9	14.1
				Cultured 146⁻	89.6	59.3	32.8	10.5
				Cultured J11d⁻	87.5	52.5	28.5	11.0
2	MHC-incompatible (CxD2)	B6xD2	Spleen	Cultured FcR⁻		159.1	99.1	33.8
				Cultured N418⁺		174.7	80.8	20.8
				Fresh N418⁺		109.7	45.1	12.8
			Thymus	Cultured FcR⁻		218.2	163.4	91.4
				Cultured N418⁺		229.3	157.7	68.4
				Fresh N418⁺		136.3	43.0	10.8
	Mls-incompatible (CxB6)	B6xD2	Spleen	Cultured FcR⁻		181.2	74.6	20.6
				Cultured N418⁺		186.3	69.7	17.3
				Fresh N418⁺		170.7	43.0	10.8
			Thymus	Cultured FcR⁻		134.7	52.7	12.6
				Cultured N418⁺		117.7	35.1	7.2
				Fresh N418⁺		38.7	7.2	2.1

CD4⁺ T cells were stimulated with different preparations of dendritic cells. Cultured denditic cells were cells that became nonadherent after overnight culture of low-density adherent cells. These were further purified by rosetting with antibody-coated sheep erythrocytes (FcR⁻), by the treatment with mAb (RA3-3A1 anti-B220a or J11d anti-heat-stable antigen) plus complement, or by sorting with FACStar plus as N418⁺ cells from cultured dendritic cells or from freshly prepared cell suspensions (25). The purity of the dendritic cell preparations in Exp. 1 was monitored by LPS-induced proliferation. The values of [³H]thymidine incorporation between 58 and 70 h culture at 2 × 10⁵ cells/well with 20 μg/ml LPS were 4,800 cpm, in cultured cells. 1,018 cpm in FcR⁻, 206 cpm in 146⁻, and 248 cpm in J11d⁻ of BALB/c dendritic cells, and 5,219, 1,197, 309, and 276 cpm in CxD2 dendritic cells, respectively. Spleen cells under the same culture condition incorporated [³H]thymidine of 184,950 cpm. T cell poliferation was monitored by [³H]thymidine incorporation between 72 and 84 h of culture. Each number represents the mean of triplicate cultures.
* Dose of APC.

response to Mls is mediated by this subset (28). Thymic B cells proved to be strong stimulators of an anti-Mls response (Fig. 1 *A* [◇]), whereas resting splenic B cells were weak (Fig. 1 *A* [▽]), as reported (18). Mls-1ᵃ-bearing, CxD2 F_1 thymic B cells were at least 100 times more potent in stimulating BALB/c T cells than syngeneic CxD2 B cells (Fig. 1 *A* [compare ◇ and ◆]). The activity of the thymic B cell preparation was reduced by >90% by treatment with anti-CD5 mAb and complement (Fig. 1 *A* [◇ vs. ▮]). Splenic B cells had to be activated by anti-Ig to become strong APC for Mls antigens (Fig. 1 *A* [□]), again as reported (18). In contrast to previous results, however, it appeared that thymic and spleen dendritic cells could present Mls antigens. Specifically, we found that T cells proliferated more actively when there was an Mls disparity expressed by the dendritic cell (CxD2-stimulating BALB/c) than when there was no such disparity (BALB/c-stimulating BALB/c) (Fig. 1 *A* [○ vs. ●

Table 2. *Selective Stimulation of $V_\beta 6^+$ T Cells by Mls-1ᵃ*

Stimulus	Exp.	Large cells		Small cells	
		$V_\beta 8^+$	$V_\beta 6^+$	$V_\beta 8^+$	$V_\beta 6^+$
		%		%	
MHC	1	20.0	13.6	16.5	12.3
Mls	1	25.8	55.9	9.2	10.2
	2	26.5	55.3	19.8	9.8
None	1	ND	ND	19.0	13.3

Frequency of $V_\beta 6$- and $V_\beta 8$-bearing small and large cells, as distinguished by forward light scatter (Fig. 2), in BALB/c CD4⁺ T cells that were unstimulated or stimulated with MHC- vs. Mls-incompatible dendritic cells (Fig. 2).

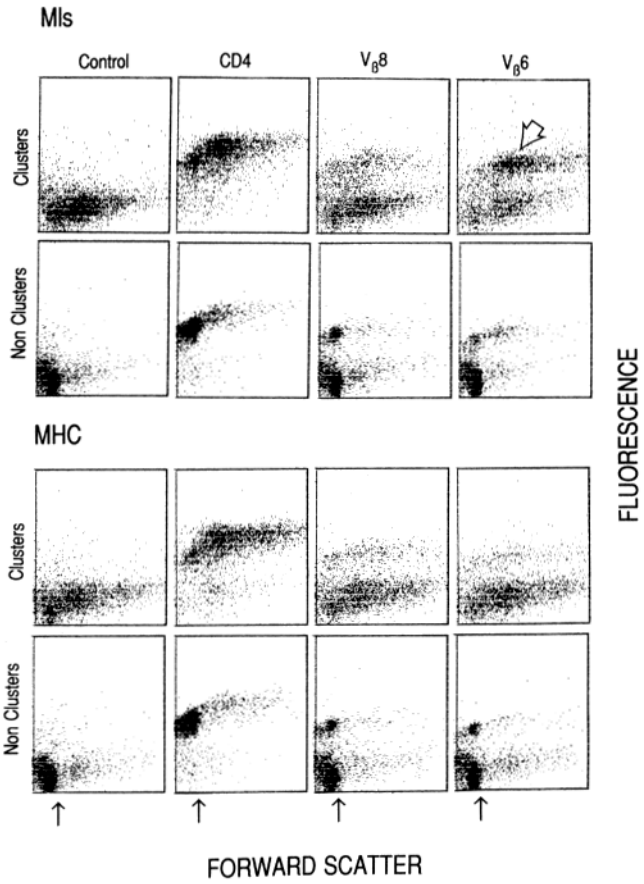

Figure 2. Frequency of $V_\beta 8^+$ and $V_\beta 6^+$ T cells during the response of BALB/c T cells to dendritic cells from Mls-incompatible, MHC-compatible BALB/c × DBA/2 or MHC-incompatible B6.H-2k mice. After 3 d of culture in which 30,000 dendritic cells were used to stimulate 300,000 $CD4^+$ T cells, the cultures were harvested and stained with rat mAb followed by FITC mouse anti-rat Ig. The rat mAb are listed at the top and include a nonreactive control (RB6 antigranulocyte), anti-CD4 (GK1.5), anti-$V_\beta 8$ (KJ16), and anti-$V_\beta 6$ (44-22-1). Dead cells and debris were gated out on the basis of propidium iodide-staining and low forward light scatter. Shown here are the data on live cells comparing fluorescence (*y*-axis, four decates) and forward light scatter (*x*-axis) as a measure of cell size. The arrows denote the location of small unstimulated lymphocytes, while to the right of the arrow are found the enlarged blasts. To show that the blasts were responding to dendritic cells, the cultures were separated into cluster and noncluster fractions, since most of the dendritic cells and blasts are found in large aggregates as described (44). Note that most of the large blasts are found in the cluster fraction when either Mls or MHC is the stimulus (e.g., *open arrow*). However, in the case of Mls, the majority of the blasts express the Mls-1-reactive TCR, $V_\beta 6$, whereas in the case of MHC, the frequency of $V_\beta 6$ blasts is low and comparable with unstimulated T cells (latter not shown).

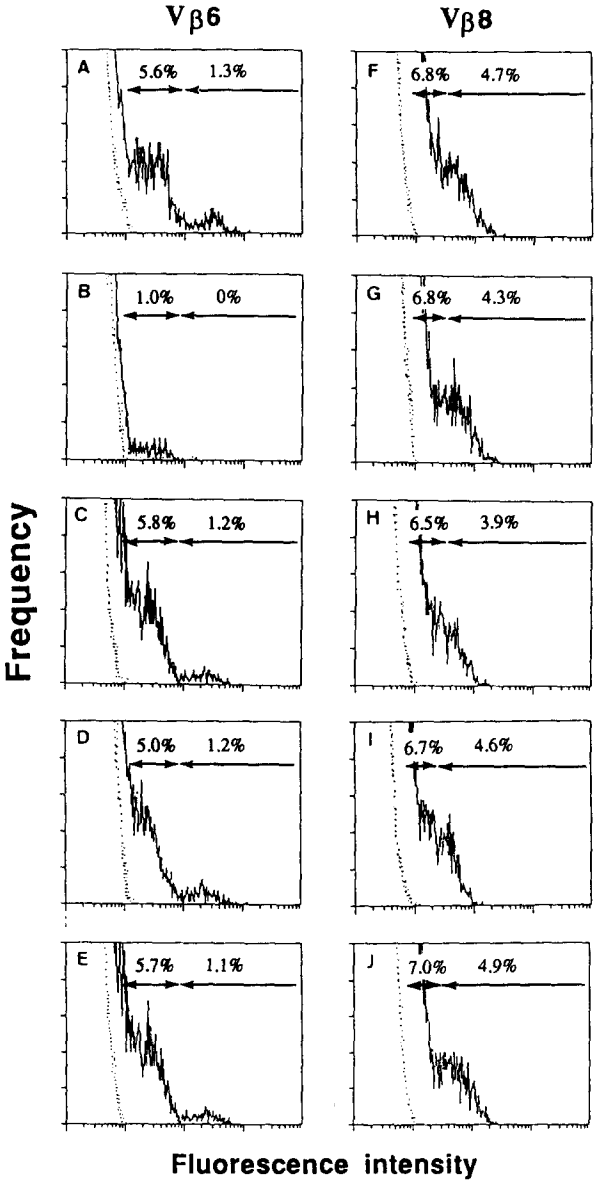

Figure 3. Deletion of $V_\beta 6^+$ T cells in BALB/c mice by intrathymic injection of thymic B cells, but not splenic dendritic cells. Thymus cell suspensions from BALB/c newborn mice were stained with anti-$V_\beta 6$ mAb and FITC-conjugated anti-rat Ig Ab (*A–E*) or anti-$V_\beta 8$ mAb and FITC-conjugated anti-mouse Ig Ab (*E–J*) 7 d after injection with CxD2 thymic B cells (*B* and *G*), thymic dendritic cells (*C* and *H*), high density spleen B cells (*D* and *I*), or splenic dendritic cells (*E* and *J*). $V_\beta 6$ and $V_\beta 8$ expression in thymocytes from BALB/c newborn mice without injection are shown as controls (*A* and *F*) and are similar to results obtained after injection of syngeneic BALB/c APC (Fig. 4 *A*). Dead cells were gated out by the staining with ethidium bromide.

and △ vs. ▲]). APC from both CxD2 and BALB/c mice actively stimulated $CD4^+$ T cells from MHC-incompatible C57BL/6 mice (Fig. 1 *B*).

It is possible that the proliferation of T cells that was induced by dendritic cells was due to a small contamination of Ig^+ B cells in the dendritic cell population. However, B cell–depleted purified dendritic cell preparations were comparably effective in stimulating Mls-disparate T cells to conventional FcR^- dendritic cell preparation (Table 1, Exp. 1).

In addition, positively selected cultured $N418^+$ ($CD11c^+$) dendritic cells (25) from the spleen and the thymus were also a potent stimulator for Mls-disparate $CD4^+$ T cells (Table 1, Exp. 2). Fresh $N418^+$ spleen dendritic cells appeared to be slightly less active than cultured cells as described for stimulation of the primary MLR (25). This was more evident in thymic dendritic cells.

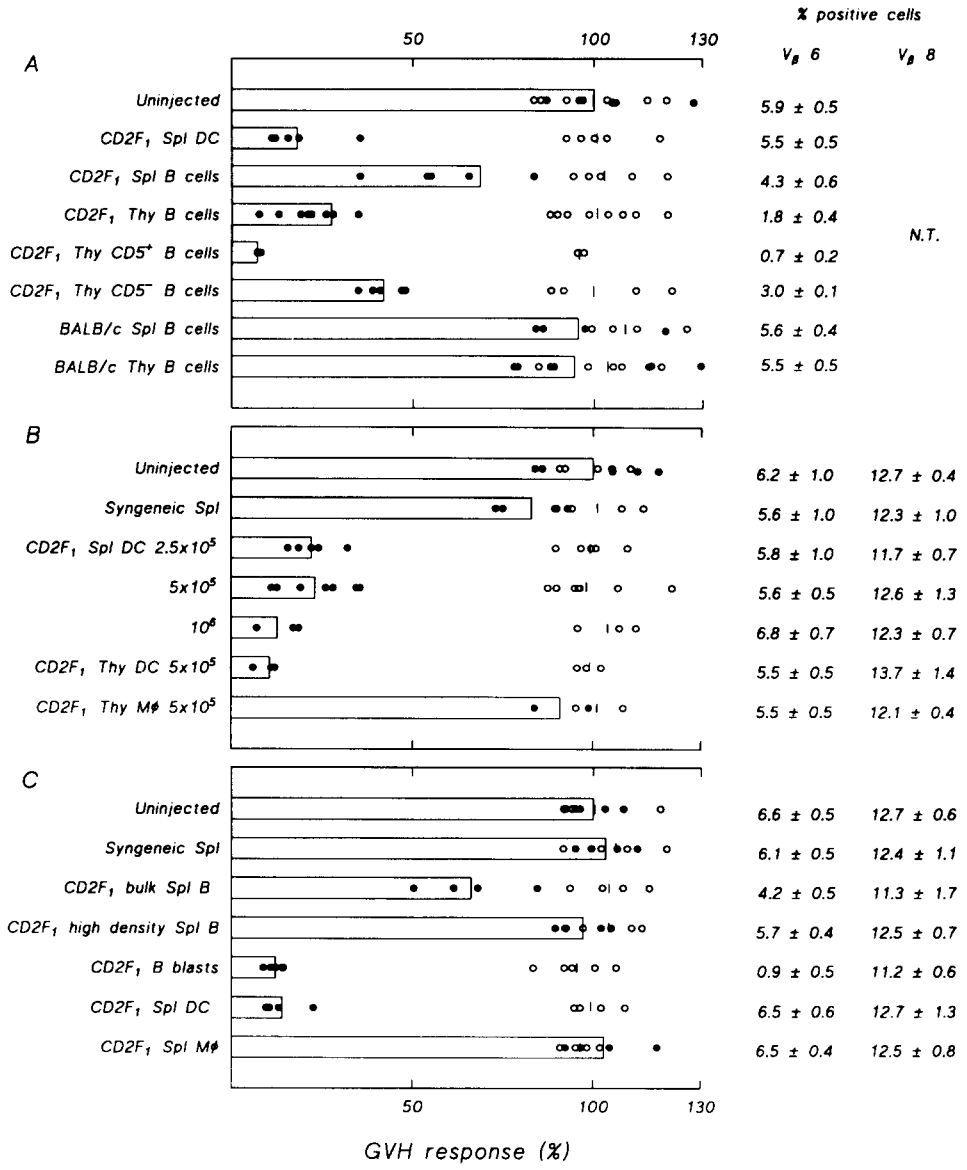

Figure 4. In vivo reactivity to Mls-1^a antigen of thymocytes from mice injected with various APC. Neonatal BALB/c mice were given intrathymic injections of 5 × 10^5 APC (*A* and *C*), or the indicated numbers of dendritic cells from spleen or thymus (*B*). Responsiveness of the thymocytes to Mls-1^a antigen (CxD2, *filled circles*) and MHC antigen (C3H, *open circles*) at 1 wk after injection was measured by lymph node swelling in local GVHR. Results are shown as the mean of percent responses (*open bar* and *short vertical line*) relative to that induced by thymocytes from age-matched uninjected BALB/c newborn mice. Each circle represents the response of an individual mouse. The values of lymph node weight in six mice as positive and negative controls (see Materials and Methods) in anti-Mls response and anti-MHC response were 3.02 ± 0.26 and 1.09 ± 0.22 mg, and 3.43 ± 0.29 and 1.18 ± 0.20 mg in Exp. *A*; 3.23 ± 0.26 and 1.02 ± 0.11 mg, and 3.55 ± 0.32 and 1.22 ± 0.19 mg in Exp. *B*; and 3.20 ± 0.31 and 0.99 ± 0.13 mg, and 3.68 ± 0.29 and 1.19 ± 0.16 mg in Exp. *C*, respectively. The response to Mls and MHC antigens in mice injected with medium alone was 1.03 ± 0.24 and 1.11 ± 0.08 mg in *A*, 0.97 ± 0.12 and 1.13 ± 0.15 mg in *B*, and 0.96 ± 0.11 and 1.14 ± 0.15 mg in *C*, so that the differences between medium control and the negative injection control were insignificant. A part of the thymocytes from each mouse was stained with anti-Vβ6 mAb (44-22-1) plus FITC anti-rat Ig or anti-Vβ8 (F23.1) plus FITC anti-mouse Ig for FACS analysis. Values are the sum of percentage of Vβ6^{high} and Vβ6^{low} T cells, and results represent the mean values with SD. Expression of Vβ8 was not determined in *A*, but the percent positive cells of Vβ6 and Vβ8 in separate experiments were 5.9 ± 0.1 and 11.6 ± 0.5 in uninjected BALB/c, 0.7 ± 0.4 and 11.3± 0.3 in mice injected with CxD2 thymic B cells, 3.0 ± 0.4 and 11.3 ± 0.3 in mice injected with CxD2 CD5$^-$ thymic B cells, and 5.8 ± 0.1 and 11.3 ± 0.2 in mice injected with CxD2 splenic dendritic cells.

That the stimulating antigens on dendritic cells were in fact Mls was also evidenced by the finding that −55% of the enlarged T blasts induced by Mls-disparate CxD2 dendritic cells expressed the Vβ6 gene that confers Mls reactivity, whereas only −13% of the blasts were positive after stimulation with MHC-incompatible dendritic cells (Fig. 2 and Table 2). Therefore, stimulation with Mls selectively expands T cells expressing Vβ6, while stimulation with MHC does not expand these cells above their normal frequency. Only a slight increase in the frequency of Vβ8$^+$ blasts was observed after stimulation by Mls-disparate dendritic cells relative to allogenic dendritic cells (Table 2). This result suggests that Vβ6$^+$ T cells are the major cell type responding to Mls-1^a antigen, although some Vβ8.1, 7, and 9 T cells can also be reactive from work in other laboratories. Similar results were obtained in Mls-MLR using thymic dendritic cells, thymic B cells, and splenic B blasts. We therefore conclude that thymic B cells, splenic B blasts, and both thymic and spleen dendritic cells can carry Mls antigens in a stimulatory form.

Thymic B Cells Induce Neonatal Tolerance by a Deletional Mechanism. We proceeded to inoculate both lobes of neonatal thymi with populations of thymic and splenic B cells and dendritic cells. The injected APC were from CxD2 F_1 mice (Mls-1^a), or from syngeneic BALB/c as controls, and the neonates were 0–1-d-old BALB/c [Mls-1^b], so that we could monitor the frequency of Vβ6-bearing T cells that responded to Mls-1^a. We evaluated the thymi at day 7 when

Table 3. *Anergy of T Cells after Injection of Mls-incompatible Dendritic Cells*

Treatment of mice	Mouse no.	Percent $V_\beta6^+$ cells	Response [^{3}H]TdR uptake to stimulation with: No mAb	Anti-$V_\beta6$	Anti-$V_\beta8$	Anti-CD3
				$\times 10^{-3}$ cpm		
None	1	5.8	960	7,615	11,189	53,600
	2	6.9	1,012	8,414	12,269	49,658
	3	6.1	1,050	7,958	13,570	55,108
CxD2 Thymic B cells	4	0.1	860	1,112	10,236	51,000
	5	0.3	1,100	1,324	11,193	53,326
	6	0.2	970	1,208	11,869	45,826
CxD2 Dendritic cells	7	5.2	1,005	2,218	12,186	50,169
	8	6.0	963	2,516	11,092	47,819
	9	5.7	1,181	2,318	10,816	51,219

Lymph node cells from axillary, inguinal, brachial and mesenteric nodes were obtained from individual mice and depleted of B cells by the treatment with mAb (TIB146; anti-B220) plus complement. Responsiveness to immobilized anti-$V_\beta6$, anti-$V_\beta8$, and anti-CD3 was assessed in vitro. Proliferation was measured by [^{3}H]TdR incorporation of 3×10^5 cells at 68–72 h for anti-$V_\beta6$ and anti-$V_\beta8$ mAbs and at 34–48 h for anti-CD3 mAb. Results represent the mean of duplicate cultures. $V_\beta6$ expression of T cells was also monitored by FACScan, as described in Fig. 2.

control BALB/c thymocytes included 5.6% $V_\beta6^{low}$ and 1.3% $V_\beta6^{high}$ T cells (Fig. 3 *A*). When the BALB/c thymi had been injected with CxD2 thymic B cells, however, there was virtually a complete deletion of both $V_\beta6^{high}$ and $V_\beta6^{low}$ (Fig. 3 *B*). Moreover, the intrathymic injection of BALB/c thymic B cells did not affect the $V_\beta6$ expression of thymocytes (not shown). Injections of resting B cells from spleen, and dendritic cells from spleen or thymus, did not delete thymocytes expressing $V_\beta6$ (Fig. 3, *C–E*). Therefore, thymic $CD5^+$ B cells can delete self-reactive T cells in neonatal thymi, but dendritic cells cannot.

To pursue tolerance at the functional level, we relied on an in vivo approach. An in vitro assay was not used, since it is difficult to induce a significant proliferative response to Mls-1^a in thymocytes. This is presumably because the number of $CD4^+$, $V_\beta6^+$ cells is so small. However, it is known that thymocytes can induce an anti-Mls response in vivo in the form of a local GVHR, in which T cells injected into the footpad lead to the enlargement of the draining popliteal lymph node (27). The thymocytes from newborn BALB/c mice given intrathymic inoculation of CxD2 Mls-incompatible APC were therefore injected into the footpads of CxD2 mice to examine the reactivity to Mls. As expected, when bulk thymic B cells or purified $CD5^+$ thymic B cells had been injected into the thymi, the thymocytes lacked Mls-1^a-reactive cells and did not induce GVH (Fig. 4 *A*). However, no change of reactivity to third-party allogenic MHC antigen was observed. Treatment of thymic B cells with anti-CD5 mAb and complement depleted some but not all of the tolerizing activity. This may indicate a role for $CD5^-$ thymic cells, or a lack of complete killing of the active $CD5^+$ component. If bulk populations of splenic B cell were injected, the change of $V_\beta6$ expression and reactivity to Mls-1^a was slight (Fig. 4, *A* and *C*). High-density resting spleen B cells induced neither a change of $V_\beta6$ expression nor tolerance to the Mls product (Fig. 4 *C*). Like thymic B cells, however, activated spleen B blasts deleted $V_\beta6$-expressing thymocytes, resulting in a lack of reactivity to Mls (Fig. 4 *C*).

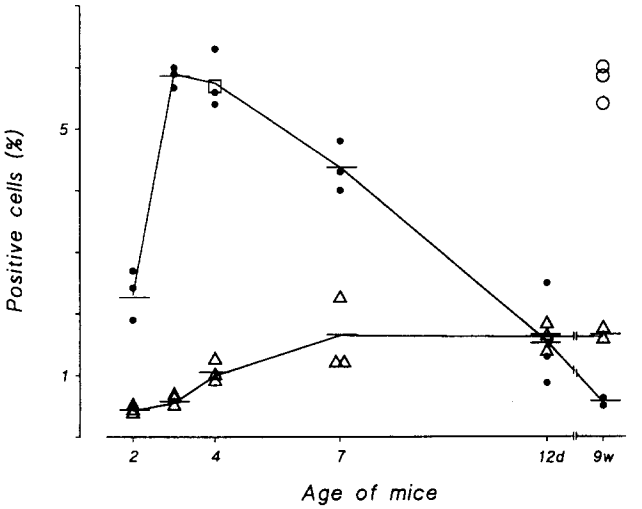

Figure 5. $V_\beta6$-expressing cells and $CD5^+$ B cells in the thymus of CxD2 mouse during the early postnatal period. Expression of $V_\beta6$ in thymocytes and the appearance of $CD5^+$ B cells in the thymus were monitored by FACS. Values from each mouse are shown for $V_\beta6^+$ cells (●) and $CD5^+$ B cells (△). The percent $V_\beta6$-expressing T cells in peripheral blood of the CxD2 mouse (□) was also determined using pooled blood from six mice. Adult BALB/c mice (○) were used as a positive control. Note that in the Fig. 3 legend, it was shown that at day 7, $V_\beta6$-expressing cells in the 7-d-old CxD2 mouse were inactive in inducing local GVHR.

Table 4. *Direct Effect of Mls-1^a-expressing Thymic B Cells on the Deletion of $V_\beta 6^+$ T Cells in the Thymus*

Exp.	Newborn mouse: Strain	Injected with: Donor	Injected with: Cell type	No.	Local GVH reaction from thymocytes injected into: BALB/c H-2^d Mls-1^b	CxD2 H-2^d Mls-1^a	B6xD2 H-2$^{b/d}$ Mls-1^a	AKR H-2^k Mls-1^a	Surface expression in lymph nodes of: $V_\beta 6$	$V_\beta 8$
1	C57BL/6	B6xD2	Thymic B	1	4.6	14.1	17.0	98.1	1.2	18.9
				2	8.1	14.8	19.8	105.6	0.9	17.7
				3	11.0	6.8	15.2	120.0	1.4	18.8
				4	4.9	9.9	2.3	95.4	1.5	18.4
			Dendritic cell	1	1.2	5.2	1.7	90.5	11.5	19.9
				2	4.9	13.0	2.3	106.7	12.2	18.9
				3	4.9	11.7	6.8	110.0	11.0	19.5
				4	11.8	19.5	15.7	106.7	13.7	19.1
		CxB6	Thymic B	1	15.9	21.0	25.8	101.2	11.2	19.8
				2	2.1	15.9	29.8	110.0	11.4	18.1
				3	4.9	17.9	30.4	98.9	12.7	14.0
				4	17.5	34.1	49.3	102.5	11.0	17.5
			Dendritic cell	1	11.3	11.1	11.1	102.2	11.9	19.3
				2	21.0	20.5	21.5	120.0	12.8	20.7
				3	1.6	2.3	5.8	111.1	12.1	18.3
				4	14.3	18.9	15.8	97.4	11.0	19.8
									Surface expression in thymocytes of:	
2 (A)					C57BL/6	CxD2	B6xD2	C3H	$V_\beta 6$	$V_\beta 8$
	BALB/c	B6xD2	Thymic B	1	10.7	8.5	14.7	99.1	0.8	8.2
				2	8.7	5.9	11.0	102.0	0.6	10.2
				3	12.9	10.0	12.6	112.7	1.1	8.0
				4	7.5	9.6	14.5	89.9	0.9	9.7
			Dendritic cell	1	10.8	18.1	12.9	89.2	7.9	8.2
				2	5.7	10.5	8.2	117.9	7.9	8.5
				3	12.6	20.3	9.6	98.6	6.5	9.5
				4	19.5	33.1	12.6	117.4	6.8	8.7
(B)					BALB/c		B6xD2	C3H		
	C57BL/6	B6xD2	Thymic B	1	15.0		17.0	121.9	0.8	9.2
				2	13.1		14.8	115.6	1.0	12.3
				3	18.7		20.1	106.5	1.3	11.5
				4	9.0		5.7	87.9	1.1	10.7
				5	5.4		4.8	97.2	0.7	11.9
				6	19.0		19.8	120.8	1.2	10.7

(continued)

Table 4. *(continued)*

Exp.	Newborn mouse: Strain	Injected with: Donor	Cell type	No.	Local GVH reaction from thymocytes injected into: BALB/c	B6xD2	C3H	Surface expression in thymocytes of: $V_\beta 6$	$V_\beta 8$
			Dendritic cell	1	18.7	24.3	104.6	7.1	11.9
				2	22.5	19.7	96.8	6.4	10.8
				3	8.2	11.3	99.4	6.9	12.7
				4	11.5	12.0	114.7	5.4	12.1
				5	9.2	5.6	112.0	6.0	10.9
				6	2.3	6.6	88.5	6.8	11.2
				7	7.4	9.3	125.1	7.1	11.6

Groups of four to seven neonatal mice were given an intrathymic injection of 5×10^5 APC per lobe. Responsiveness of the thymocytes to Mls-1^a and MHC antigens at 1 wk after injection was measured by lymph node swelling in local GVHR. All the data are given in terms of percent of the positive control value using thymoctyes from uninjected mice. The values of lymph node weight in five mice as positive and negative controls were 2.98 ± 0.18 and 0.97 ± 0.15 mg in BALB/c mice, 3.12 ± 0.23 and 1.06 ± 0.12 mg in CxD2 mice, 3.03 ± 0.18 and 0.89 ± 0.17 mg in B6xD2 mice and 3.48 ± 0.31 and 1.23 ± 0.14 mg in AKR mice (Exp. 1); and 3.02 ± 0.18 and 0.99 ± 0.12 mg in C57BL/6 mice, 2.88 ± 0.21 and 0.89 ± 0.20 mg in CxD2 mice, 3.21 ± 0.25 and 0.96 ± 0.21 mg in B6xD2 mice, and 3.54 ± 0.33 and 1.25 ± 0.24 mg in C3H mice (Exp. 2 *A*); and 3.03 ± 0.22 and 1.12 ± 0.23 mg in BALB/c mice, 2.99 ± 0.27 and 1.09 ± 0.24 mg in B6xD2 mice, and 3.56 ± 0.37 and 1.26 ± 0.28 mg in AKR mice (Exp. 2 *B*). Expression of $V_\beta 6$ and $V_\beta 8$ was monitored in a part of the bulk population of thymocytes (Exp. 2) or lymph node cells (mixture of axillary, inguinal, brachial, and mesenteric nodes) (Exp. 1) from each mouse.

Dendritic Cells Induce Neonatal Tolerance to Mls via Clonal Anergy. As shown above, splenic dendritic cells did not induce clonal deletion in neonatal mice, but surprisingly, these APC did induce functional tolerance. No local GVHR was observed using T cells from thymi that had been injected with dendritic cells, in spite of the presence of cells expressing $V_\beta 6$ (Fig. 4 *B*). This indicates that dendritic cells can induce tolerance, but the mechanism is likely to be clonal inactivation rather than deletion.

To verify that the $V_\beta 6^+$ cells in dendritic cell–tolerated mice are anergic, we tested their responsiveness to stimulation with mAb to $V_\beta 6$ and other TCR epitopes (Table 3). The lymph node cells from tolerant animals were indeed hyporesponsive to anti-$V_\beta 6$ but were normally responsive to anti-CD3 and $V_\beta 8$.

To compare other types of bone marrow–derived thymic APC with B cells, we injected thymic dendritic cells or macrophages into neonatal thymi (Fig. 4 *B*). Only the dendritic cells tolerized the mice, and as in the case of spleen dendritic cells, $V_\beta 6$ cells were not deleted. It is important to recall that to obtain thymic dendritic cells and macrophages, it is necessary to use collagenase-digested thymi (23), whereas thymic B cell isolates are obtained from teased thymi and have few other types of APC (16).

Direct Effect of Thymic B Cells on the Deletion of $V_\beta 6^+$ T Cells in Association with MHC Antigen. Deletion of $V_\beta 6^+$ T cells in the thymus is known to require the expression of MHC class II I-E molecules (14, 29, 30). To examine the possibility that elimination of $V_\beta 6^+$ T cells by thymic $CD5^+$ B cells was due to the transfer of the Mls product to host thymic dendritic cells, we injected thymic B cells or spleen dendritic cells into thymi of either I-E^- or I-E^+, Mls-1^{a-}, MHC-disparate mice (Table 4). Although both I-E^+, Mls-1^{a+} thymic B cells and dendritic cells induced tolerance to Mls-1^a, only thymic B cells deleted $V_\beta 6^+$ T cells in lymph nodes and thymi, even when injected into I-E^- mice. The same result was obtained by injecting activated B blasts (not shown), i.e., Mls-1^a, I-E^+ B blasts deleted $V_\beta 6^+$ T cells in I-E^- mice. These results demonstrate that Mls-expressing thymic B cells are able to delete developing $V_\beta 6^+$ T cells without the aid of I-E products on host APC.

Ontogeny of Thymic $CD5^+$ B Cells and Mls-reactive T Cells in Thymi. To assess if $CD5^+$ B cells might delete Mls-1^a-reactive T cells during normal thymus development, we monitored the numbers of $V_\beta 6^+$ T cells and $CD5^+$ B cells during the first week of life in Mls-1^a mice, since it is known that $V_\beta 6^+$ cells appear transiently (31). In fact, the numbers of $CD5^+$ B cells began to increase just at the time the $V_\beta 6^+$ cells began to decrease or be deleted (Fig. 5). In a separate experiment, we obtained the following data by teasing a thymic lobe; $1.3–1.5 \times 10^7$ cells from a 1-d-old CxD2 mouse, $3.3–4.5 \times 10^7$ cells from a 3-d-old mouse, and $1.1–1.4 \times 10^8$ cells from a 7-d-old mouse, in which the numbers of thymic B cells were $4–6 \times 10^4$, $3.2–3.6 \times 10^5$, and $1.05–1.55 \times 10^6$, respectively. Similar numbers of total thymocytes and thymic B cells were also obtained in BALB/c mice (data not shown).

The thymocytes from 4-d-old CxD2 mice already showed a 65–90% reduced response to Mls-1^a antigen, when assessed by local GVHR. Almost no reactivity to Mls-1^a was detected in thymocytes at day 7, as in the case of adult CxD2 mice (not shown).

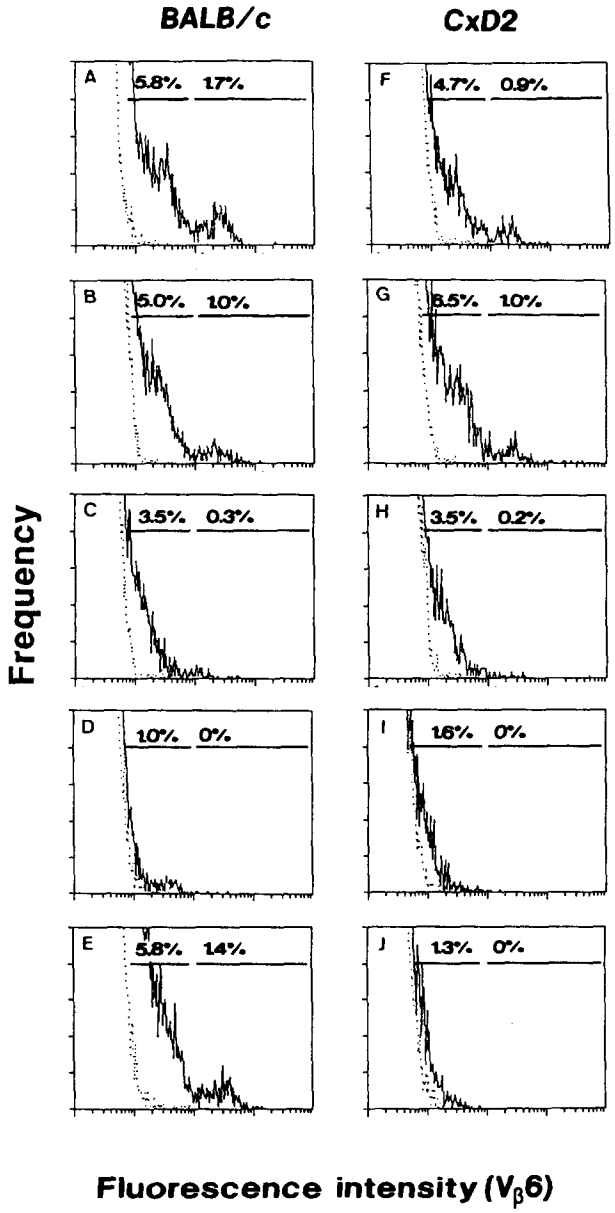

Figure 6. Similar decreasing pattern of $V_\beta 6^{high}$- and $V_\beta 6^{low}$-expressing T cells in the thymus of early postnatal CxD2 mouse and new born BALB/c mouse injected with graded dose of thymic B cells. Expression of $V_\beta 6$ was monitored in a newborn BALB/c mouse given intrathymic injection of 5×10^4 (*B*), 1.5×10^5 (*C*) or 5×10^5 thymic B cells (*D*), and a CxD2 mouse at age of 2 d (*F*), 4 d (*G*), 7 d (*H*), 14 d (*I*), and 7 wk (*J*). Age-matched uninjected (*A*) and adult (*E*) BALB/c served as controls.

We next compared the appearance of $V_\beta 6^{high}$ and $V_\beta 6^{low}$ T cells between CxD2 at various ages and BALB/c injected with graded dose of thymic B cells (Fig. 6). A low dose of thymic B cells induced a slight reduction of $V_\beta 6^{high}$, but not $V_\beta 6^{low}$, T cells (Fig. 6 *B*). A medium dose of B cells considerably deleted not only $V_\beta 6^{high}$ but also $V_\beta 6^{low}$ T cells (Fig. 6 *C*). On the other hand, the percentages of $V_\beta 6^{high}$ and $V_\beta 6^{low}$ T cells in CxD2 at 7 d were less than those in age-matched BALB/c (Fig. 6, *H* vs. *A*), and both decreased gradually with age (Fig. 6, *G–J*). Finally, only a small number of $V_\beta 6^{low}$ T cells remained in the thymus in the adult mouse (Fig. 6, *J* vs. *E*).

Discussion

It has been demonstrated by Mazinger and Guerder (32) that dendritic cells from spleen abrogate the generation of cytotoxic T cells in fetal thymus in vitro, indicating that the dendritic cells, the most potent activator for peripheral T cells, are also potent in the inactivation of developing T cells. The experiments presented here examine a wider range of APC and provide evidence that different types of thymic APC can have different roles in the establishment of tolerance: thymic B cells tolerize by a deletion mechanism; thymic dendritic cells tolerize by inducing anergy; and thymic macrophages are inactive. Deletion also was observed with splenic B blasts but not resting B cells. Like thymic dendritic cells, spleen dendritic cells induce anergy, while splenic macrophages are inactive.

Mls, which has not been identified biochemically, is a cell-associated antigen that is expressed gradually on B cells (17–19). Dendritic cells did not express detectable Mls previously (17, 18), whereas our results here show that both thymic and splenic dendritic cells can express Mls-1^a, as reported by Sunshine et al. (33). Mls-1^a-specific T cell proliferation induced by dendritic cells is not due to B cells that contaminate the dendritic cell preparation, since B cell depletion did not affect the stimulatory activity of dendritic cells, and highly purified $N418^+$ dendritic cells also induced T cell proliferation to Mls-1^a. This discrepancy may be ascribed to the difference of mouse strains or to the method of assay. In our case, we used CxD2 mice and monitored $V_\beta 6^+$, Mls-1^a-reactive blasts directly.

Hengartner et al. (34) have documented that T cells bearing TCR with self-reactivity are present at high density in the thymic cortex but not in the medullary region. This strengthens the idea that the deletion of self-reactive T cells takes place at the cortico-medullary junction, where bone marrow–derived dendritic cells are localized (35, 36). However, our results show that dendritic cells injected intrathymically do not induce deletion of self-reactive T cell clones. Considering that thymic B cells are detected throughout the thymus (37) and that Mls antigens are a product of activated B cells (17, 18), it may well be that the dendritic cell only acquires small amounts of Mls antigens during their preparation as a result of transfer from thymic B cells or spleen B cells (14). This idea seems to be compatible with the result that fresh $N418^+$ thymic and spleen dendritic cells are equally potent in stimulating MHC-incompatible T cells, but thymic dendritic cells are much less effective in inducing Mls-disparate T cell proliferation than spleen dendritic cells. Larger amounts of Mls antigens on dendritic cells, which might be produced by injected thymic B cells, may lead to deletion rather than anergy. This possibility remains to be elucidated.

The mechanism of clonal deletion by thymic B cells or splenic B blasts injected intrathymically is so far not clear. The APC might directly interact with developing T cells in cortex or medulla. Both dendritic cells and B cells in the

thymus are detected at 14 d of gestation (38–42). B cells increase in number after birth by day 7. Preceding this, the number of $V_\beta 6^+$ T cells in the CxD2 mouse reaches a maximum at day 3–4 and then decreases. These results seem to support the idea that Mls products secreted by thymic B cells are taken up by dendritic cells, and then, Mls-reactive T cells are clonally deleted. It has been documented that tolerance to Mls results from the selective depletion of $V_\beta 6^{high}$ T cells (34), and that the class II I-E molecules are required for the presentation of the Mls-1^a product to $V_\beta 6$-expressing T cells in the thymus (29, 30). However, thymic B cell–induced tolerance was accompanied by the deletion of not only $V_\beta 6^{high}$ but also $V_\beta 6^{low}$-expressing T cells. A similar phenomenon was observed even when thymic B cells were injected into Mls-1^{a-}, I-E^- C57BL/6 mice. In addition, a low dose of thymic B cell induced the incomplete but preferential decrease of $V_\beta 6^{high}$ T cells, whereas a medium dose significantly reduced both $V_\beta 6^{high}$ and $V_\beta 6^{low}$ cells. This was also the case when CxD2 thymic B cells were injected into neonatal C57BL/6 thymi (data not shown). Therefore, our conclusion for the time being is that thymic B cells by themselves can, after intrathymic injections, directly interact with developing $V_\beta 6^{low}$-positive T cells and delete them.

Recently, Webb and Sprent (43) reported that $CD8^+$ T cells play a role in the clonal deletion of Mls-reactive T cells in the thymus. They inoculated the cells into newborn mice intravenously via the facial vein. Small numbers of injected cells migrated into the thymus, and the tolerant state lasted for >7 wk. We cannot exclude the possibility that small numbers of $CD8^+$ T cells contaminate our preparation of dendritic cells or thymic B cells. Unlike their report, however, thymic dendritic cells or B cells injected intrathymically induced temporary tolerance that lasted only 9–10 d and was not detectable at 14 d both in the thymus and in lymph nodes (data not shown). Therefore, dendritic cells and B cells, which express the class II MHC molecules needed to present Mls, may differ in the mechanism of tolerance induction from $CD8^+$ T cells that do not express class II.

The direct, neonatal intrathymic injection protocol described here may prove useful in delineating the role of specific types of APC in the induction of self-tolerance. A role of thymic B cells, including $CD5^+$ B cells, might be considered in further analyses of this important area of thymus physiology. Our results apply to the widely studied Mls-1^a antigen, and need to be extended to other antigens.

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