T Cell Contact with Ia Antigens on Nonhemopoietic Cells In Vivo Can Lead to Immunity Rather than Tolerance

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Summary

Long-term H-2-heterozygous $a \rightarrow (a \times b)F_1$ bone marrow (BM) chimeras prepared with supralethal irradiation (1,300 rad) are devoid of Ia^+ host BM-derived antigen-presenting cells (APC), but show quite strong host Ia expression in germinal centers, probably on follicular dendritic cells (a class of nonhemopoietic stromal cells). To examine whether Ia expression on these non-BM-derived cells is capable of inducing post-thymic tolerance of T cells, thymectomized irradiated $(a \times b)F_1$ mice were reconstituted with parent a stem cells and then, 6 mo later, given parent a thymus grafts. As measured by primary mixed lymphocyte reactions and V_β expression, the $CD4^+$ cells differentiating in the thymus-grafted mice showed no detectable tolerance to the H-2 (Ia) antigens of the host. To examine whether the thymus-grafted mice contained immunologically significant quantities of host Ia antigens, long-term $a \rightarrow (a \times b)F_1$ chimeras were injected with normal strain a $CD4^+$ cells; the donor cells were recovered from thoracic duct lymph of the chimeras and tested for host reactivity in vitro. The results showed that Ia expression in the chimeras was sufficient to cause selective trapping of a substantial proportion of host-Ia-reactive $CD4^+$ cells soon after transfer and, at later stages, to induce strong priming. Tolerance was not seen. The data place constraints on the view that T cell recognition of antigen expressed on cells other than typical BM-derived APC leads to tolerance induction.

T cell tolerance to MHC molecules occurs largely in the thymus (1, 2). Tolerance reflects deletion of immature T cells encountering MHC molecules displayed on marrow-derived APC, and, to a lesser extent, on epithelial cells. The possibility that T cells can also be tolerized to MHC antigens in the post-thymic environment is suggested by the finding that transgenic mice in which MHC molecules are expressed selectively in the β cells of the pancreas (3, 4) show marked tolerance to the transgenic MHC molecules. These and other findings (5) imply that contact of mature T cells with MHC molecules expressed on cell types other than typical APC can lead to tolerance rather than immunity.

To investigate this question, we constructed thymus-grafted mice under conditions designed to allow newly formed strain a $CD4^+$ T cells to make post-thymic contact with strain b H-2 (Ia) antigens expressed selectively on non–bone marrow–derived (NBMD)[1] cells. In a second model, mature strain a $CD4^+$ cells were exposed to Ia^+ $(a \times b)F_1$ NBMD cells on adoptive transfer. No tolerance was seen in either model; indeed, conspicuous priming resulted in the second model. The data would thus appear to contradict the dogma that T cell contact with antigen on "nonprofessional" APC leads to tolerance.

Materials and Methods

Mice. Mice were bred at the breeding facility of the Research Institute of Scripps Clinic.

Preparation of Thymus-grafted Mice. $(B6 \times CBA/J)F_1$ mice were thymectomized (Tx) at 6–8 wk of age (6), exposed to heavy irradiation (1,300–1,400 rad) 2–3 wk later, and then, within 4 h, reconstituted intravenously with T-depleted bone marrow (BM) cells ($\sim 5 \times 10^6$ BM cells treated with anti-Thy-1 mAb + C) (7); 5 wk or 6 mo later, the chimeras were grafted under the kidney capsule with 1-d-old donor vs. host thymuses (three per mouse) exposed to 1,100 rad in vitro (8). The mice were kept in positive-pressure isolators and given antibiotics in the drinking water. The mice were killed at 9–10 wk post-grafting to prepare T cells.

Monoclonal Antibodies. mAbs specific for Thy-1.2 (J1j, rat), heat-stable antigen (J11d, rat), CD4 (GK1.5, rat), CD8 (3.168.8, rat), $V_\beta 11$ (RR3-15, rat), $V_\beta 8.1 + 8.2$ (KJ16-133, rat), and $I\text{-}A^{k(r, f, s)}$

[1] *Abbreviations used in this paper:* BM, bone marrow; BMC, bone marrow chimeras; BMR, bone marrow reconstituted; DC, dendritic cells; FDC, follicular dendritic cells; NBMD, non–bone marrow–derived; TDL, thoracic duct lymph; TG, thymus grafts; Tx, thymectomized.

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(10.2.16, mouse) were obtained and used as described elsewhere (7). We also used mAbs specific for Thy-1.1 (19E12, mouse) (9), $V_\beta5$ (MR9-4, mouse) (J. Bill et al., manuscript in preparation), I-E^k (14.4.4, mouse) (10), and SM3G11 (3G11) (mouse) (11).

Preparation of BM Chimeras. Chimeras used for histological analysis or as hosts for parental strain $CD4^+$ cells were prepared by exposing (B6 × CBA/J)F_1 or (B6 × CBA/Ca)F_1 mice to 1,300 rad followed by reconstitution intravenously with ~5 × 10^6 T-depleted B6 or B6.PL BM cells (7). All mice were left for at least 6 mo before testing. After 6 mo, some chimeras were given a second dose of irradiation (900–1,000 rad) followed by further reconstitution with parental strain BM cells (7).

IFN-γ Treatment In Vivo. Chimeras and control mice were given four intraperitoneal injections of murine rIFN-γ (80,000 U/injection) at daily intervals. Mice were used at day 4 after the first injection. IFN-γ was kindly provided by Ryuji Maekawa (Shionogi Research Laboratories, Osaka, Japan).

Blood-to-Lymph Recirculation of T Cells through Irradiated Hosts. Using a modification of a technique described elsewhere (12, 13), long-term chimeras and control mice were exposed to 900 rad and then, 4–6 h later, injected intravenously with a dose of ~8 × 10^7 purified B6 $CD4^+$ cells (LN cells treated with a mixture of anti-CD8 and J11d mAb + C [14]). Thoracic duct cannulas were inserted in the mice 16 h later and thoracic duct lymph (TDL) was collected continuously on ice for several days. Lymph-borne cells from two to four mice/group were washed and then stained for surface markers or used as responder cells for MLR. Host-derived cells in the lymph were quite rare (usually <10%).

Mixed Lymphocyte Reactions. Using purified $CD4^+$ cells as responder cells, MLR were set up under standard conditions as described elsewhere (14).

FACS® Analysis. To detect cells expressing V_β TCR molecules, lymphoid cells were stained with anti-$V_\beta11$ (rat) and anti-$V_\beta8$ (rat) TCR mAbs followed by FITC-labeled H and L chain–specific affinity-purified $F(ab')_2$ fragments of mouse anti–rat IgG (Pel-Freez Biologicals, Rogers, AR), and with anti-$V_\beta5$ (mouse) mAb followed by FITC-labeled goat anti–mouse IgG (Fc fragment specific) (Cappel Laboratories, Malvern, PA). In most experiments, the cells were then stained with PE-labeled anti-CD4 mAb (Becton Dickinson & Co., Mountain View, CA). For staining for $V_\beta11$ vs. 3G11, cells were incubated with biotinylated anti-$V_\beta11$ mAb and unconjugated 3G11 mAb followed by FITC-conjugated rat anti–mouse IgG (H + L chain specific) (Jackson ImmunoResearch, West Grove, PA) and PE-conjugated streptavidin (Biomedia, Foster City, CA). Cells were analyzed on a FACS IV® flow cytometer (Becton Dickinson & Co.).

Staining of Cryostat Sections. Freshly removed organs were quickly frozen in liquid nitrogen, and 5–6-μ sections were cut with a cryostat. The sections were briefly dried, fixed in acetone for 2 min, and incubated with optimal concentrations of biotinylated antibodies for 1 h. After washing, horseradish peroxidase–conjugated streptavidin (Jackson ImmunoResearch) was added for an additional 30 min, washed, and incubated with the substrate 3-amino-9-ethylcarbazole (Sigma Chemical Co., St. Louis, MO) (0.1 μg/ml in 0.05 M NaOAc pH 5.2) with 0.01% H_2O_2 for 20 min. The stained sections were then washed and photographed.

Results

To examine the tolerogenicity of Ia antigens expressed on NBMD cells, parent *a* BM cells were allowed to differentiate in parent *a* thymus grafts (TG) placed in Tx heavily irradiated H-2 heterozygous (*a* × *b*)F_1 hosts. This model rests on the assumption that parent → F_1 bone marrow chimeras (BMC) prepared with heavy irradiation express significant levels of host Ia on stromal (NBMD) cells but lack host-derived BM-derived cells. The extent of host Ia expression in BMC is considered below.

Host Ia Expression in a → (a × b)F_1 BM Chimeras. When (*a* × *b*)F_1 mice are exposed to supralethal irradiation, e.g., 1,300 rad, and reconstituted with parent *a* BM cells, Ia^+ (H-2 class II^+) host cells with APC function in vitro disappear rapidly (7). By 2 mo post-transfer, virtually all functional APC in suspensions of spleen and thymus are of donor origin. Host Ia expression in cryostat sections of spleen from a chimera tested at 6 mo post-reconstitution is shown in Fig. 1. Chimeras were constructed by transferring T-depleted B6.PL ($H\text{-}2^b$) BM cells to (B6 × CBA)F_1 ($H\text{-}2^b$ × $H\text{-}2^k$) mice exposed to 1,300 rad. Cells expressing a high density of host I-A^k or I-E^k molecules were not seen in the splenic red pulp and were conspicuously absent from the periarteriolar lymphocyte sheaths (PALS) of the spleen and the marginal sinuses of the white pulp borders, i.e., the regions occupied by dendritic (interdigitating) cells (DC) and macrophages, respectively (Fig. 1, *D–F*). Likewise, high-density I-A^{k+} cells were undetectable in LN, lung, liver, gut, and epidermis (data not shown). Nevertheless, moderately high I-A^k and I-E^k expression was observed in the central regions of primary follicles and germinal centers. Since a similar pattern of staining was observed in twice-irradiated chimeras given a total dose of 2,200 rad (see Materials and Methods), it is unlikely that the Ia^{k+} cells in primary follicles and germinal centers were BM derived. The staining in these sites was probably restricted to follicular dendritic cells (FDC) (15). These cells are not of hematogenous origin (16) and express significant though variable levels of Ia molecules (16, 17). FDC are not related to typical BM-derived DC.

In addition to FDC, a low but variable level of host Ia expression was evident on blood vessel endothelial cells of the chimeras, especially in the red pulp. Ia expression on these cells was markedly elevated in chimeras injected with rIFN-γ (80,000 U daily for 4 d) (Fig. 1, *G* and *H*).

The above findings confirm that parent → F_1 chimeras prepared with supralethal irradiation are apparently devoid of host-derived Ia^+ BM-derived cells but do show significant Ia expression on certain NBMD cells, especially FDC and endothelial cells.

Antihost Reactivity of $CD4^+$ T Cells Developing in Tx (a × b)F^1 Mice Given a BM Cell and a TG. Tx (B6 × CBA)F^1 mice were exposed to heavy irradiation (1,300 rad) and reconstituted with T-depleted $Thy\text{-}1.1^+$ B6.PL ($H\text{-}2^b$) BM. After 5 wk, these Tx, irradiated, BM-reconstituted (Tx.Ir.BMR) mice received irradiated (1,100 rad) $Thy\text{-}1.2^+$ B6 vs. (B6 × CBA)F_1 neonatal TG. Primary MLR by B6.PL-derived ($Thy\text{-}1.2^-$) $CD4^+$ T cells prepared from these mice at 10 wk post-grafting are shown in Table 1. It can be seen that, both for LN cells and mature thymocytes, the $H\text{-}2^b$ $CD4^+$ cells from mice given host-type (B6 × CBA)F_1 TG showed strong tolerance to host $H\text{-}2^k$ APC but responded well to third-party bm12 APC (group 3). By contrast, $CD4^+$ cells from mice with $H\text{-}2^b$ TG gave definite responses to $H\text{-}2^k$

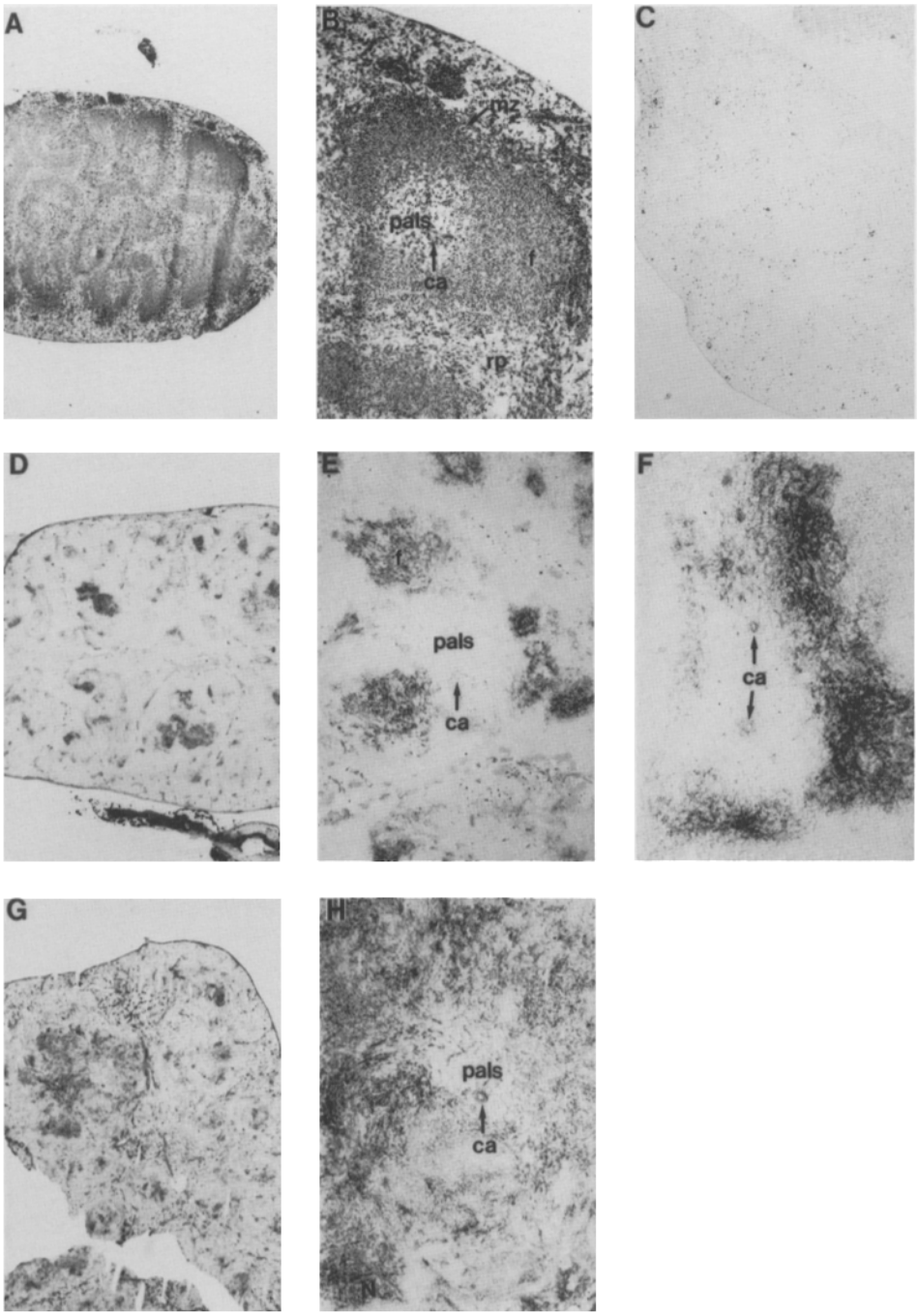

Figure 1. Host Ia expression in spleens of (B6 × CBA)F_1 and long-term B6.PL → F_1 BMC. Cryostat sections of spleens from a normal (B6 × CBA)F_1 mouse, and a 6-mo B6.PL → F_1 BMC were stained for expression of 1-A^k and I-E^k, as described in Materials and Methods. (*A*) Low power (×40) view of normal F_1 spleen stained for I-A^k; staining is evident throughout the spleen. (*B*) High power (×100) view of *A*: staining is evident in the red pulp (rp), the marginal zone (mz), and the follicles (f), and periarteriolar lymphocyte sheaths (pals) of the white pulp; the central arteriole (ca) is arrowed. (*C*) Low power (×40) view of B6 (I-A^b) spleen stained for I-A^k; except for the punctate staining of cells in the red pulp (indicative of cells with endogenous peroxidase activity), no staining is apparent. (*D*) Low power (×40) view of B6.PL → F_1 BMC spleen stained for I-A^k; patchy staining in the white pulp and striate staining of red pulp sinusoids is evident. (*E*) High power (×100) view of *D*; staining of the white pulp is limited to focal areas in follicles; there is no staining in the pals or mz. (*F*) High power (×200) view of spleen from B6 PL → F_1 BMC stained for I-E^k; the pattern of staining is essentially the same as in *E* (note that the magnification is higher for *F* than for *E*, and that *F* and *E* involved different fields). (*G*) Low power (×40) view of spleen from B6.PL → F_1 BMC pretreated with IFN-γ (Materials and Methods); strong staining is evident throughout the spleen. (*H*) High power (×100) view of *G*; strong staining of stromal cells (but not hemopoietic cells) is seen throughout the white pulp.

APC (group 4); though significant, these responses were nevertheless considerably lower than the control responses of normal H-2^b $CD4^+$ cells (group 1).

To examine whether the partial tolerance seen in the above mice reflected incomplete disappearance of host APC after irradiation, the interval between BMR and TG was extended from 5 wk (Table 1) to 6 mo (Table 2). Under these conditions, the LN $CD4^+$ cells differentiating in H-2^b TG showed no detectable tolerance to the H-2^k antigens of the host (Table 2). The same findings applied to mature $CD4^+$ (8^-) cells prepared from the TG of the chimeras (Table 3). These data on TG mice contrasted with the strong tolerance seen in control parent → F_1 chimeras, i.e., where the $CD4^+$ cells differentiated in the endogenous thymus of the host (Table 2, bottom group).

Recently, we observed that $CD4^+$ cells from B6 (I-E^-) → (B6 × CBA)F_1 (I-E^+) chimeras show considerable (50–70%) deletion of $V\beta11^+$ cells (7); in normal mice, $V_\beta11^+$ cells are selectively deleted in I-E^+ mice. $V\beta11$ expression on T cells from the 6-mo TG mice discussed above are shown in Table 4. It can be seen that, in marked contrast to normal (B6 × CBA)F_1 mice, $V_\beta11^+$ cells were not

Table 1. *Primary MLR by CD4⁺ Cells Generated in Tx.Ir.BMR (H-2^{b}xH-2^k)F$_1$ Mice Given H-2^b Stem Cells Followed 5 wk Later by H-2^b vs. F$_1$ Thymus Grafts*

Group	CD4⁺-enriched responders (10^5)	Tissue source of responders	[³H]TdR incorporation with stimulators: B6.PL (H-2^b)	CBA (H-2^k)	bm12 (H-2^{bm12})	Response to H-2^k/ response to H-2^{bm12}
			cpm × 10³			
1	Normal B6.PL	LN	1.5	146.5	80.8	1.8
		Thymus	0.3	49.7	69.4	0.7
2	Normal (B6 × CBA)F$_1$	LN	2.3	3.0	125.8	< 0.1
		Thymus	0.2	0.3	56.4	< 0.1
3	B6.PL BM → Tx.Ir (B6 × CBA)F$_1$	LN	0.2	3.1	60.0	< 0.1
	with (B6 × CBA)F$_1$ TG	Thymus	0.2	0.4	35.2	< 0.1
4	B6.PL BM → Tx.Ir (B6 × CBA)F$_1$	LN	0.6	26.4	51.7	0.5
	with B6 TG	Thymus	0.2	28.3	68.2	0.4

Tx.Ir. BMR mice were prepared as described in Materials and Methods and the text. To ensure that the T cells generated in the Tx.Ir.BMR mice were not contaminated with radioresistant cells present in the TG at the time of grafting, the mice received Thy-1.1⁺ BM cells (B6.PL) and Thy-1.2⁺ TG. Purified CD4⁺ CD8⁻ T cells depleted of Thy-1.2⁺ cells were prepared from LN and the thymus of the hosts at 10 wk post-grafting and used as responder cells (10^5) in MLR with irradiated spleen cells (5×10^5) as stimulators. The data show mean responses for triplicate cultures harvested on day 4. Note that, to prevent responses to Mls^a determinants, CBA/Ca rather than CBA/J stimulators were used.

Table 2. *Primary MLR by CD4⁺ Cells Generated in Tx.Ir.BMR (H-2^b × H-2^k)F$_1$ Mice Given H-2^b Stem Cells Followed 6 mo Later by H-2^b Thymus Grafts: MLR by CD4⁺ Cells from LN*

CD4⁺-enriched responders (2×10^5)	Tissue source of responders	Day of MLR	MLR: [³H]TdR incorporation with stimulators: B6.PL (H-2^b)	(B6 × CBA)F$_1$ (H-2^{bxk})	bm12 (H-2^{bm12})	Response to H-2^k/ response to H-2^{bm12}
				cpm × 10³		
Normal B6.PL	LN	3	2.3	37.4	42.6	0.9
		4	4.4	76.4	64.5	1.2
Normal (B6 × CBA)F$_1$	LN	3	1.4	1.9	33.0	<0.1
		4	2.9	3.8	78.4	<0.1
B6.PL BM → Tx.Ir (B6 × CBA)F$_1$ + B6 TG	LN	3	2.5	73.6	61.9	1.2
		4	4.4	76.4	64.5	1.2
B6.PL → Ir (B6 × CBA)F$_1$ (non-Tx)	LN	3	1.1	5.2	45.9	0.1
		4	2.3	15.4	95.5	0.1

Tx.Ir.BMR (B6 × CBA)F$_1$ mice were prepared as described in Table 1, except that thymus grafting was delayed for 6 mo after irradiation and BM reconstitution. Cell suspensions from LN were prepared at 9 wk after thymus grafting. Control parent → F$_1$ chimeras were made by exposing normal (euthymic) (B6 × CBA)F$_1$ mice to 1,300 rad and reconstituting these mice with B6 BM cells; 3 mo later, these mice received further irradiation (1,000 rad) followed by reconstitution with B6.PL BM; these mice are abbreviated "B6.PL → Ir (B6 × CBA)F$_1$" in the table. As in Table 1, all cell suspensions were treated with anti-Thy-1.2 mAb + C before use. Primary MLR were assayed as for Table 1, except that a higher dose of responder cells (2×10^5) was used.

Table 3. *Primary MLR by CD4⁺ Cells Generated in Tx.Ir.BMR (H-2^b × H-2^k)F$_1$ Mice Given H-2^b Stem Cells Followed 6 mo Later by H-2^b Thymus Grafts: MLR by CD4⁺ Cells from LN vs. Thymus*

CD4⁺-enriched responders (2 × 10^5)	Tissue source of responders	Day of MLR	MLR: [^{3}H]TdR incorporation (cpm × 10^3) with stimulators: B6.PL (H-2^b)	CBA (H-2^k)	bm12 (H-2^{bm12})	Response to H-2^k/ response to H-2^{bm12}
Normal B6.PL	LN	3	3.4	86.7	41.7	2.1
		4	4.1	219.0	89.3	2.5
	Thymus	5	0.1	49.0	20.5	2.4
Normal (B6 × CBA)F$_1$	LN	3	2.3	2.4	43.2	< 0.1
		4	3.5	5.3	82.7	< 0.1
	Thymus	5	0.3	0.1	28.6	< 0.1
B6.PL BM → Tx.Ir (B6 × CBA)F$_1$ + B6 TG						
Mouse no. 1	LN	3	2.2	62.0	29.7	2.2
		4	3.3	176.9	72.0	2.5
Mouse no. 2	LN	3	2.7	112.4	32.4	3.7
		4	3.8	158.0	77.5	2.1
Mouse nos. 1 + 2	Thymus	5	0.1	104.6	44.6	2.4

As for Table 2, except that the CD4⁺ cells were prepared both from LN and the TG.

Table 4. *V$_\beta$11 Expression on LN T Cells Generated in Tx.Ir.BMR (H-2^b × H-2^k)F$_1$ Mice Given H-2^b Stem Cells Followed 6 mo Later by H-2^b Thymus Grafts*

Mice tested	No. of mice tested	Percent of CD4⁺ cells expressing: V$_\beta$11	V$_\beta$8	Percent of CD8⁺ cells expressing V$_\beta$11	V$_\beta$8
Normal B6.PL	3	4.0	15.9	9.6	16.0
		4.3	15.4	9.0	17.6
		3.6	17.2	10.8	17.6
	Mean =	4.0	16.2	9.8	17.1
Normal (B6 × CBA)F$_1$	3	0.0	14.4	2.7	14.2
		0.5	15.8	2.8	15.7
		0.0	15.1	5.3	15.3
	Mean =	0.2	15.1	3.6	15.4
B6.PL BM → Tx.Ir. (B6 × CBA)F$_1$ with B6 TG	4	7.6	25.6	12.5	22.1
		5.2	20.0	13.6	27.3
		5.6	20.8	17.6	29.5
		3.9	23.0	14.2	24.4
	Mean =	5.6	22.4	14.5	25.9

LN T cells from normal mice and the TG mice used in Tables 2 and 3 were double stained for V$_\beta$11 (RR3-15) or V$_\beta$8.1+8.2 (KJ16 mAb) vs. CD4 or CD8 and analyzed by flow cytometry using two-channel immunofluorescence (Materials and Methods). For the TG mice, the data were calculated with respect to Thy-1.1⁺ cells.

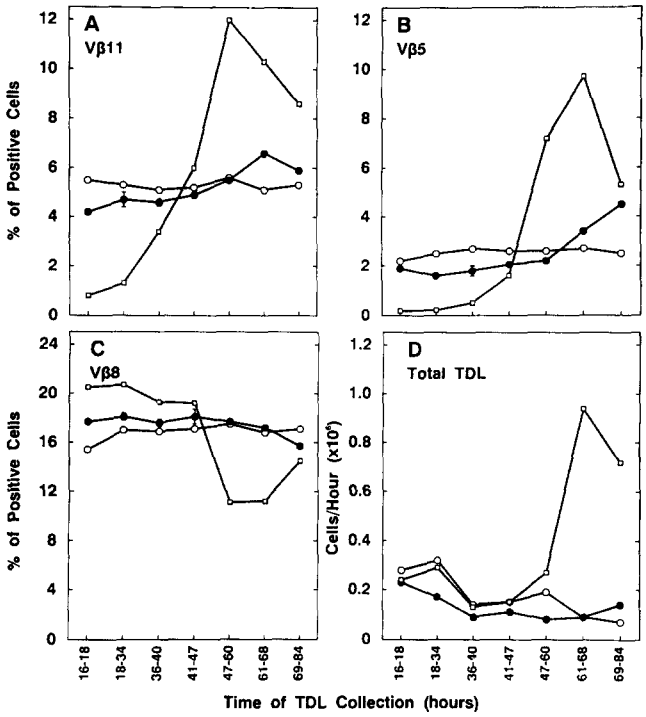

Figure 2. V_β expression on thoracic duct cells collected from irradiated B6.PL mice (○), (B6 × CBA)F_1 mice (□), and B6.PL → F_1 BMC (●) injected with normal B6 CD4+ cells. All mice were exposed to 900 rad 4 h before intravenous injection of 8 × 10^7 B6 CD4+ cells (LN cells treated with J11d + anti-CD8 mAb + C); thoracic duct cannulas were inserted 14 h later, and lymph was collected continuously for the intervals shown. CD4+ cells in TDL were analyzed for expression of V_β11 (*A*), V_β5 (*B*), V_β8 (*C*), and for total numbers (*D*). V_β expression on CD4+ cells was assessed by dual fluorescence and FACS® analysis (Materials and Methods). Mean values for individual mice (two to three mice/group) are shown. It can be seen that B6 CD4+ cell transfer to B6.PL (I-E−) hosts led to no change in V_β ratios; cell outputs in TDL declined progressively and nearly all of the lymph-borne cells were small lymphocytes. B6 CD4+ cell transfer to normal F_1 (I-E+) hosts led to an initial marked decline in $V_\beta11^+$ and $V_\beta5^+$ cells followed by an increase in these cells. This increase corresponded with the appearance of blast cells in the lymph; by 60 h, nearly all of the lymph-borne cells were blasts. The decrease in $V_\beta8^+$ cells at 47–68 h presumably means that these cells were underrepresented in the host-reactive early blast populations; the later rise in $V_\beta8^+$ cells at 69–84 h may be an indication of the marked over-representation of $V_\beta8^+$ cells seen at 2 wk post-transfer (see Fig. 6). With B6 CD4+ cell transfer to the chimeras, there was a minor decrease in $V_\beta11^+$ and $V_\beta5^+$ cells in early lymph collections and a slight increase in these cells in later collections; total cell outputs declined progressively, although blast cells were apparent in late lymph collections. In the particular experiment illustrated, all three groups of mice were treated with IFN-γ before T cell injection. Based on other experiments (see text), IFN-γ treatment did not enhance the degree of V_β selection seen in the chimeras or potentiate the production of blast cells. It should be noted that in all three groups of mice, the vast majority of the lymph-borne cells were donor-derived (Thy-1.2+, H-2^k−) CD4+ cells.

deleted in Tx.Ir.BMR (B6 × CBA)F_1 hosts given H-2^b BM plus H-2^b TG.

Recognition of Host Ia Antigen in Parent → F_1 Chimeras by Normal T Cells. One explanation for the lack of functional tolerance in the TG mice is that host Ia expression in these mice was simply too low to be relevant. To address this question, we used the technique of blood-to-lymph recirculation of normal T cells through irradiated hosts (13, 18–21). This technique traps host-reactive T cells in the lymphoid tissues, presumably as a manifestation of T cell binding to host H-2 bearing cells. Trapping is manifested by a transient disappearance of host-reactive T cells from TDL. In normal immunogenic donor/host combinations, this stage of negative selection is maximal at 1–2 d post-transfer. Thereafter, the progeny of the trapped cells proliferate extensively and re-enter TDL in large numbers as blast cells: the stage of positive selection.

For the experiments discussed below, B6.PL → 1,300 rad (B6 × CBA)F_1 (F_1) chimeras were exposed to 900 rad at 6 mo post-transfer and injected 2–4 h later with a large dose (~8 × 10^7) of purified CD4+ cells prepared from normal B6 mice; as controls, B6 CD4+ cells were also transferred to irradiated normal F_1 and B6.PL mice. In some of the experiments, the host mice were pretreated with IFN-γ for 3 d before irradiation and T cell transfer. Thoracic duct cannulation was performed ~16 h after T cell injection, and TDL were collected continuously over the next 4–5 d. The vast majority (≥90%) of the lymph-borne cells were of donor (Thy-1.2+) origin.

V_β Expression. With I-E− → I-E+ strain combinations, negative and positive selection of host-I-E-reactive T cells is demonstrable by monitoring V_β11 expression on the donor T cells entering TDL (13). As shown in Fig. 2, negative and positive selection of $V_\beta11^+$ cells and also $V_\beta5^+$ cells was conspicuous when B6 CD4+ cells were transferred to normal F_1 hosts. The proportions of $V_\beta11^+$ and $V_\beta5^+$ cells were approximately eightfold below normal in early lymph collections (16–34 h) but then rose to two- to threefold above normal levels in later collections (47–68 h) before declining; $V_\beta8^+$ cells, which are not I-E reactive, showed reciprocal kinetics. No alteration in V_β expression occurred when B6 CD4+ cells were transferred to syngeneic B6.PL hosts; here, the proportions of $V_\beta11^+$, $V_\beta5^+$, and $V_\beta8^+$ cells in the lymph remained constant throughout the 4-d drainage period. In contrast to normal F_1 hosts, negative and positive selection of $V_\beta11^+$ and $V_\beta5^+$ cells was quite limited when B6 CD4+ cells were transferred to the chimeras. In a total of four experiments, including two with mice pretreated with IFN-γ, the proportions of $V_\beta11^+$ and $V_\beta5^+$ cells in lymph of the chimeras were reduced by 10–20% in early collections and increased by 10–30% in late collections. The best evidence for selection is shown in the experiment illustrated in Fig. 2 in which the hosts were IFN-γ pretreated. Based on other experiments, however, it did not appear that IFN-γ pretreatment enhanced selection. The point to emphasize is that negative and positive selection of $V_\beta11^+$ and $V_\beta5^+$ (but not $V_\beta8^+$) cells was reproducibly demonstrated in the chimeras but was clearly much less marked than in normal F_1 hosts.

Activation Markers on Lymph-borne Cells. With transfer of B6 CD4+ cells to normal F_1 hosts, the re-appearance of $V_\beta11^+$ and $V_\beta5^+$ cells in TDL after the stage of negative selection was associated with a marked (~10-fold) increase in total numbers of cells in the lymph (Fig. 2 *D*). By day 3, nearly all of these cells were blast cells. With T cell transfer

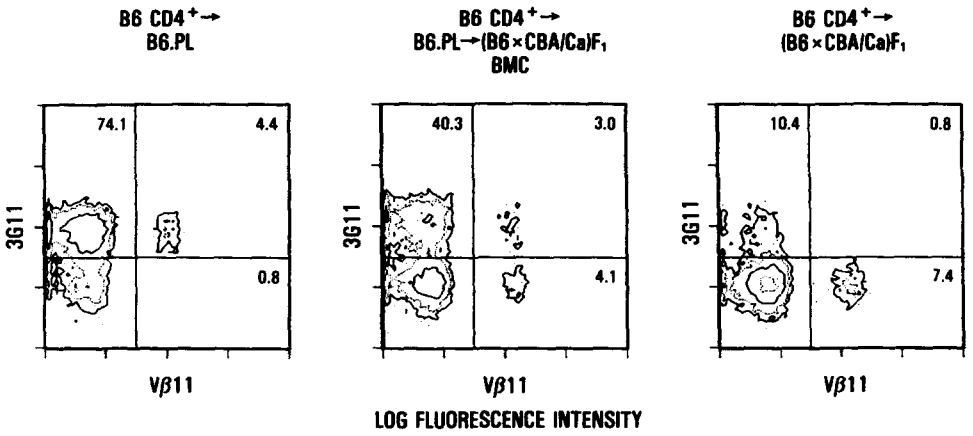

Figure 3. Vβ11 vs. 3G11 expression on late collections of thoracic duct cells from the three groups of mice described in Fig. 2. The lymph-borne cells were collected at 69–84 h post-transfer. Cells were stained for Vβ11 vs. 3G11 (a marker on resting CD4+ cells) by dual fluorescence as described in Materials and Methods. By adding the percentages in the lower two boxes, it can be seen that the proportion of 3G11− cells, i.e., cells with an activated phenotype, was low in the CD4+ → B6.PL group (21.5%), intermediate in the CD4+ → chimera group (56.7%), and very high in the CD4+ → normal F_1 group (88.8%). Note that for the CD4+ → chimera group, 58% of the Vβ11+ cells had an activated (3G11−) phenotype. For earlier (<40 h) collections of lymph, nearly all of the cells from each group of mice were 3G11+ (data not shown).

to the chimeras, by contrast, the cell content of the lymph was no higher than in the control B6 CD4+ → B6.PL group. This applied for the first 3 d post-transfer. Thereafter, there was a slight (but reproducible) increase in TDL cell numbers in the chimeras. This increase was associated with the appearance of small numbers of blast cells; by day 4, blast cells accounted for 20–40% of the lymph-borne cells (compared with 10–20% for the B6 → B6.PL group).

Fig. 3 shows the expression of activation markers on the lymph-borne cells collected at 69–84 h from the mice tested in Fig. 2; the cells were double stained for Vβ11 vs. 3G11, a marker for virgin (resting) T cells (11). Early lymph collections from all three groups of mice consisted almost entirely of small 3G11+ cells (data not shown). For later lymph collections (Fig. 3), the cells from the B6 → B6.PL group remained 3G11+. By contrast, the blast cells (including Vβ11+ blasts) generated in the B6 → normal F_1 group were nearly all 3G11−, indicative of activated T cells. With the B6 → chimera group, 3G11− cells began to appear in the lymph at the end of day 3 and reached 60% of total cells by day 4–5. The proportion of these cells was not significantly higher in IFN-γ-pretreated chimeras. Essentially similar findings applied when the cells were stained for other markers that distinguish resting and activated T cells, e.g., Pgp-1 (data not shown).

T Cell Selection Measured by MLR. The experiments in Fig. 4 were designed to examine whether acute blood to lymph recirculation of B6 CD4+ cells through the B6.PL → F_1 chimeras was able to cause trapping of functional T cells, i.e., T cells responsive to normal F_1 stimulator cells in vitro. The donor cells were collected from the lymph at 20–40 h post-injection and used as responder cells for MLR. The results of three experiments are shown in Fig. 4 (*A* and *B*, *C–E*, *F–H*); in one of the experiments (*F–H*), the chimeras (and controls) were pretreated with IFN-γ. As expected, B6 CD4+ cells filtered through control B6.PL mice gave high MLR to normal (B6 × CBA)F_1 stimulators (I-A + I-E difference) and weaker responses to B10.A(5R) stimulators (I-E difference alone). By contrast, B6 CD4+ cells filtered through normal F_1 hosts were completely unresponsive to F_1 and 5R stimulators; responses to third-party bm12 stimulators were retained. The significant finding was that filtration of B6 CD4+ cells through the chimeras caused a partial reduction in the response to F_1 and 5R stimulators. This reduction was specific because responses to bm12 remained largely unchanged. Based on the magnitude of the MLR mediated by graded doses of responder cells, the specific reduction in the anti-F_1 and anti-5R MLR by CD4+ cells filtered through the chimeras vs. control B6.PL hosts amounted to ~50% (see Fig. 4 legend). This also applied to chimeras pretreated with IFN-γ.

MLR by the lymph-borne cells collected from the above three groups of mice at a later stage, i.e., 48–62 h post-injection, are shown in Fig. 5 and Table 5. At this stage, most of the cells in the lymph of the B6 CD4+ → normal F_1 group were blast cells (see above). These cells gave a low but significant MLR to F_1 stimulators, which peaked on day 2–3 and then declined to low levels by day 4 (Table 5). This is a typical response of recently activated T cells (21). Quite different results were seen with CD4+ cells collected from the chimeras. As with cells from the control B6 → B6.PL group, the lymph-borne cells from the chimeras gave typical primary responses to F_1 stimulators with higher responses on day 4 than on day 3 (Table 5). Significantly, in contrast to earlier lymph collections, there was no reduction in the response to F_1 (Fig. 5) or 5R (data not shown) stimulators. This was seen in three separate experiments, including one experiment with IFN-γ-pretreated mice (Fig. 5, *C* and *D*).

The above findings apply to cells recovered from the chimeras at 50–60 h post-transfer, i.e., the early stage of positive

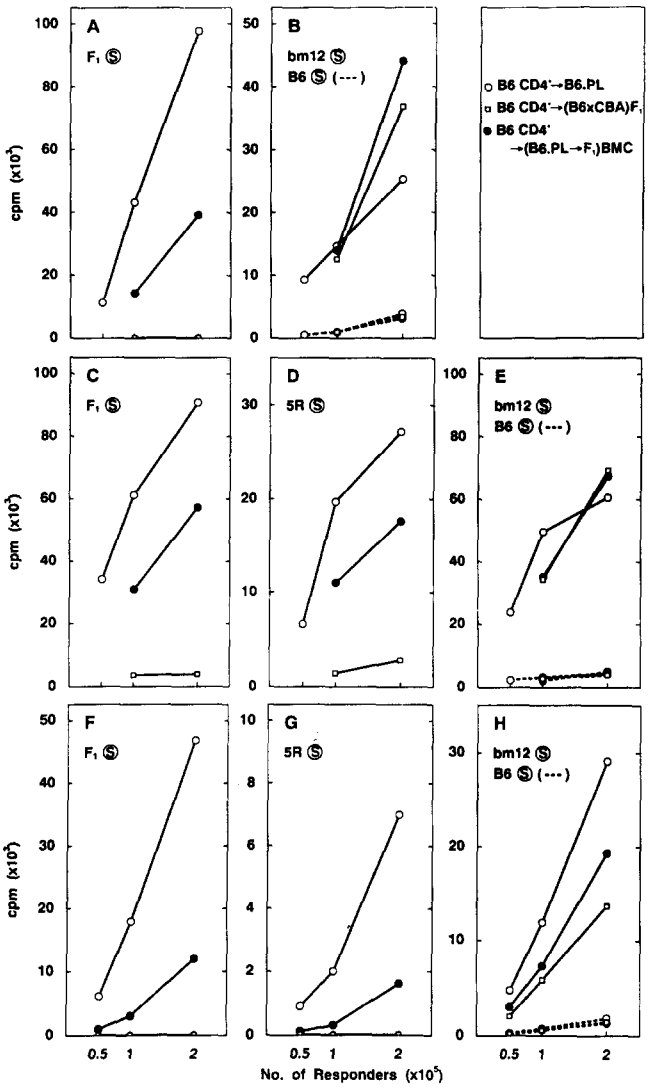

Figure 4. Acute recirculation of B6 CD4+ cells through B6.PL → F_1 chimeras causes partial depletion of cells responsive to host-type F_1 and 5R (host I-E only) spleen cells in vitro. Doses of 8×10^7 normal B6 CD4+ cells were transferred intravenously to normal B6.PL mice (○), normal F_1 mice (□), or B6.PL → F_1 BMC (●) exposed to 900 rad 4 h before (Fig. 2); the donor cells were recovered from TDL at 20–40 h post-injection. Various doses of these cells were used as responder cells for MLR using F_1, 5R, and bm12 irradiated spleen cells as stimulators (Ⓢ) (Materials and Methods); MLR were harvested on day 4 or 5. The results of three different experiments are shown (*A* and *B*, *C–E*, and *F–H*); in the third experiment (*F–H*), the mice were pretreated with IFN-γ before T cell injection. Each point represents the mean of triplicate cultures. It can be seen that filtration of B6 CD4+ cells through normal F_1 hosts reduced the MLR to F_1 and 5R by close to 100% but had little effect on the response to third-party bm12. B6 CD4+ cells filtered through the chimeras showed a partial reduction in the response to F_1 and 5R. In calculating the extent of this reduction, it is evident that the anti-F_1 and anti-5R responses mediated by a dose of 2×10^5 CD4+ cells filtered through the chimeras (e.g., in *C* and *D*) was roughly equivalent to the responses mediated by 10^5 CD4+ cells filtered through the control B6.PL mice. Taking into consideration the response to bm12 (which was reduced only in the third experiment [H]), the specific reduction in the MLR to F_1 and 5R by CD4+ cells filtered through the chimeras ranged from 40 to 60%. This reduction was not accentuated in IFN-γ-treated mice.

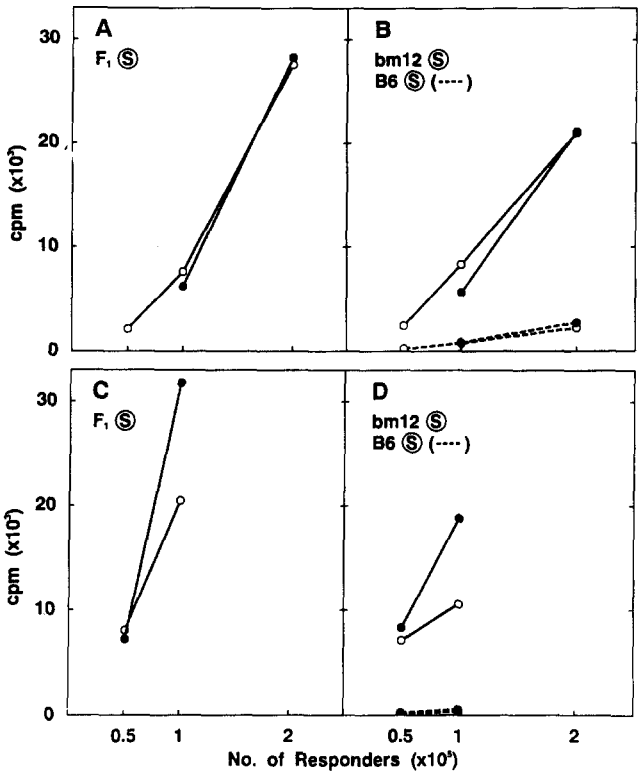

Figure 5. At day 3 after injecting irradiated B6.PL → F_1 BMC with normal B6 CD4+ cells, the donor CD4+ cells in TDL show no reduction in MLR to host-type F_1 spleen stimulators (Ⓢ). The results show two experiments (*A* and *B*, and *C* and *D*) in which TDL were collected at 48–62 h post-injection. These experiments were a continuation of the first and third experiments in Fig. 4, and the symbols used in Figs. 4 and 5 are the same. In contrast to early lymph collections (Fig. 4), it can be seen that the anti-F_1 MLR mediated by CD4+ cells collected from the chimeras at 48–62 h post injection (●) was no lower than the response by CD4+ cells passed through control B6.PL mice (○); note that, for the cells passed through the chimeras, the higher response to F_1Ⓢ in the second experiment (*C*) was associated with an equivalent increase in the response to bm12Ⓢ (*D*).

selection. Since activated ($3G11^-$) cells were rare in the lymph at this time ($3G11^-$ cells appeared in TDL of the chimeras only after 70 h, see above), it was important to study the host reactivity of cells recovered at later stages. Testing the MLR of cells collected from the chimeras at >60 h was difficult because of low yields of cells. Instead, the following approach was used. Groups of chimeras and control normal F_1 mice were exposed to sublethal irradiation (500 rad), injected with a large dose of B6 CD4+ cells, and then left for 16 d before thoracic duct cannulation. As shown in Fig. 6, purified donor B6 CD4+ cells recovered from TDL of the chimeras showed clear evidence of specific priming. MLR to third-party (bm12) stimulators were unchanged (relative to normal B6 CD4+ responders), but responses to F_1 and 5R were markedly elevated and easily detectable with a low dose of 5×10^4 responder cells (*G*, *H*, and *I*); with higher doses of responders, anti-F_1 MLR were prominent as early as day 2 of culture (*D* and *E*). The selective priming to F_1

Table 5. *MLR by B6 CD4⁺ Cells Filtered through B6 → F_1 BMC and Recovered during Early Stage of Positive Selection*

Filtration hosts (900 rad) for B6 CD4⁺ cells*	No. of responders for MLR (10^5)	MRL with stimulators: B6 d3	B6 d4	(B6 × CBA)F_1 d3	(B6 × CBA)F_1 d4	bm12 d3	bm12 d4
				cpm × 10^3			
B6.PL	1	0.7	1.0	8.1	17.4	9.0	12.9
	2	2.2	4.4	27.4	63.8	23.2	49.1
B6.PL → F_1 BMC	1	0.8	0.8	7.0	15.5	6.4	12.0
	2	2.7	4.5	31.0	62.3	23.8	49.3
(B6 × CBA)F_1	1	1.0	2.2	12.1	4.4	33.9	35.9
	2	2.4	3.1	11.6	3.4	52.4	35.6

* Cells recovered from lymph at 48–62 h post-injection.

and 5R stimulators was not associated with a significant alteration in V_β ratios (*F*). Passaging B6 CD4⁺ cells through the control normal F_1 mice gave somewhat different results. These cells gave primed responses to F_1 and 5R stimulators, but this was associated with reduced responses to bm12 (relative to control B6 CD4⁺ responders) (Fig. 6, *A* and *B*, and data not shown). Significantly, priming in normal F_1 mice led to a marked alteration in V_β ratios: proportions of $V_\beta11^+$ and $V_\beta5^+$ cells were considerably reduced whereas $V_\beta8^+$ cells were elevated (*C*), i.e., the converse of the ratios observed during early positive selection (see Fig. 2).

Discussion

In previous studies, we reported that the environment of parent → F_1 BM chimeras prepared with supralethal irradiation (1,300 rad) was strongly tolerogenic for newly formed T cells (7). The donor-derived CD4⁺ cells generated in the host thymus gave only weak MLR to normal host-type APC (spleen) in vitro and showed 50–70% depletion of host-I-E-reactive $V_\beta11^+$ T cells. Since spleen and thymocyte suspensions from the chimeras appeared to be completely depleted of host-type APC, it was argued that tolerance to host Ia antigens was induced intrathymically, presumably by thymic epithelium. The possibility that tolerance was induced extrathymically, however, could not be excluded. As shown here, host Ia expression in the spleen of long-term chimeras was not seen in sites occupied by BM-derived APC but was clearly demonstrable in germinal centers, probably on follicular dendritic cells (which are reported to be non-BM derived [16]); in IFN-γ-treated chimeras, host Ia expression was also prominent on vascular endothelium. Based on the findings of others (see Introduction), one might expect such Ia expression on "nonprofessional APC" to be highly tolerogenic, especially for newly formed T cells.

The thymus grafting experiments reported here failed to verify this prediction. Thus, when strain *a* stem cells differentiated in strain *a* TG placed in long-term Tx.Ir.BMR (*a* × *b*)F_1 mice, the *a*-derived CD4⁺ cells gave high MLR to normal host-type (*a* × *b*)F_1 spleen cells in vitro and showed no depletion of host I-E-reactive $V_\beta11^+$ cells; this contrasted with the strong tolerance seen when the chimeras received (*a* × *b*)F_1 rather than *a* TG, i.e., a situation where host Ia was encountered on thymic epithelium. These results provide an alternative explanation for the TG experiments of Bradley et al. (22). In contrast to the present study, Bradley et al. (22) observed strong host tolerance when Tx.Ir.BMR (*a* × *b*)F_1 mice were given parent *a* stem cells and a parent *a* TG. The authors concluded from this finding that T cells are susceptible to prethymic tolerance. Since the TG were applied before irradiation, however, we suggest that the TG were rapidly permeated with host BM-derived cells and that these cells induced intrathymic tolerance of newly formed T cells. We avoided this problem by leaving the Tx.Ir.BMR (*a* × *b*)F_1 mice for a prolonged period, i.e., 6 mo, before applying the TG. Grafting after a shorter interval, i.e., 5 wk, led to partial tolerance.

A trivial explanation for the lack of tolerance seen in our long-term TG mice is that host Ia expression in these mice was simply too low to be relevant. Alternatively, the CD4⁺ cells in the TG mice might have been selectively tolerized to unique self-peptide/Ia complexes expressed only on NBMD cells (23). These possibilities were assessed by injecting long-term parent → F_1 chimeras with normal parental-strain CD4⁺ cells. The expectation here was that, if host Ia expression in the chimeras were insignificant or tissue-specific, the donor CD4⁺ cells would maintain their normal pattern of blood-to-lymph recirculation and show unimpaired reactivity to the host Ia antigens expressed on normal F_1 spleen cells in vitro.

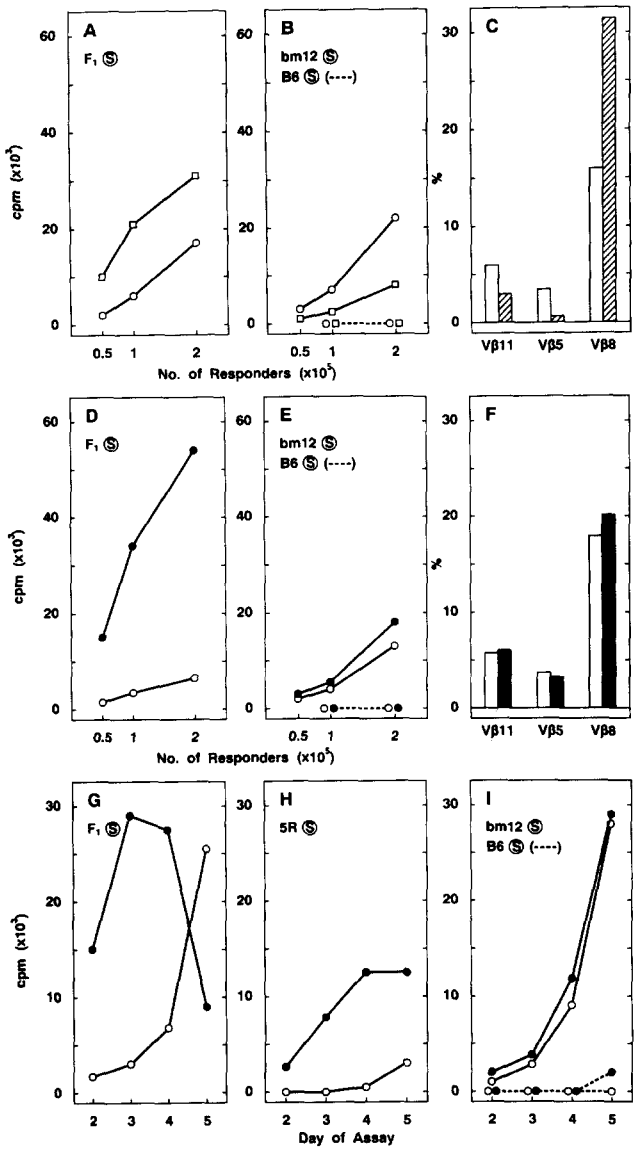

Figure 6. B6 CD4$^+$ cells transferred to B6.PL → F_1 chimeras for 2 wk exhibit marked sensitization to the host antigens. Groups of B6.PL → F_1 chimeras and normal F_1 mice were exposed to light irradiation (500 rad) and injected with a dose of 8 × 10^7 B6 CD4$^+$ cells. The two groups of mice were set up separately and were thus tested at different times. At day 16 post-transfer, the mice were cannulated to obtain TDL, and the lymph-borne cells (pooled from two to three mice/group) were used as responder cells for MLR as in Figs. 4 and 5, and also tested for V_β expression; >90% of the lymph-borne cells were donor-derived CD4$^+$ cells. (*A* and *B*) Early (day 2) MLR by normal B6 CD4$^+$ cells (O) vs. B6 CD4$^+$ cells passed through the control normal F_1 hosts (□); (*C*) V_β expression on the passaged cells (*hatched*) vs. control normal B6 CD4$^+$ cells (*open*). The lymph-borne cells gave elevated responses to F_1 (S) (relative to normal B6 CD4$^+$ cells) and reduced responses to bm12O; $V_\beta11^+$ and $V_\beta5^+$ cells were reduced whereas $V_\beta8^+$ cells were elevated. Panels (*D–F*) Day 2 MLR and V_β expression for normal B6 CD4$^+$ cells (O, *open bars*) vs. B6 CD4$^+$ cells passed through the chimeras (●, *closed bars*). Relative to the control responder cells, the response to F_1(S) was markedly elevated whereas the response to bm12(S) was near normal; V_β ratios remained unchanged. (*G–I*) A time response for the MLR by cells passed through the chimeras, using 5 × 10^4 responder cells and F_1, 5R and bm12(S). With this small (limiting) dose of responder cells, the responses to F_1(S) and 5R(S) were far higher than with the control responders. These responses reached a peak on day 3–4 (F_1) or day 4–5 (5R), whereas the response to bm12 peaked on day 5.

In practice, recognition of host Ia antigens in the chimeras appeared to be considerable. Thus, when the donor CD4$^+$ cells were recovered from TDL of the chimeras 1–2 d post-transfer and tested in vitro, the MLR to normal host-type spleen cells in vitro was specifically reduced by ~50%. A priori, one might expect this selective trapping of host-reactive CD4$^+$ cells by antigen expressed on NBMD cells to be followed by tolerance induction. This was not found. Instead, the host-reactive CD4$^+$ cells reappeared in the lymph at day 3 post-transfer. Interestingly, the lymph-borne cells collected at this time (50–60 h) showed no obvious sign of activation. This was in sharp contrast to CD4$^+$ cells transferred to normal F_1 hosts; here, the lymph at day 3 contained large numbers of blast cells that responded to host-type APC in vitro with accelerated kinetics. The implication, therefore, is that Ia expression in the chimeras was not overtly immunogenic but simply slowed the rate at which the Ia-reactive cells moved through the lymphoid tissues: after a transient period of trapping in the spleen and other organs, most of the host-reactive cells re-entered the circulation still in a resting state. This explanation is in line with the report of Ford et al. (24) that T cell trapping in vivo does not necessarily result in cell activation. In parent → normal F_1 combinations, these workers found that up to 50% of the (selectively) trapped cells failed to enter cell cycle. The subsequent fate of these cells was not studied.

Host Ia expression in the chimeras was clearly immunogenic for a small proportion of the injected CD4$^+$ cells. Activated (3G11$^-$) CD4$^+$ cells were rare in the chimeras before day 3 post-transfer but were readily detectable at later stages and accounted for 40–60% of the lymph-borne cells by day 4–5 (compared with <20% for syngeneic transfers). Progressive expansion of these cells presumably accounted for the striking degree of priming observed when CD4$^+$ cells were left in the chimeras for a 2-wk period. At this stage, the lymph-borne CD4$^+$ cells from the chimeras gave very powerful and specific responses to host Ia antigens in vitro. Indeed, the level of priming in the chimeras was as high or higher than when CD4$^+$ cells were transferred to normal F_1 hosts.

Although the kinetics and duration of CD4$^+$ cell priming in the chimeras vs. normal F_1 hosts has yet to be examined in detail, it is notable that V_β ratios in the two groups of primed cells were quite different. With the I-E$^-$ → I-E$^+$ combinations used, transfer of CD4$^+$ cells to normal F_1 hosts induced strong negative selection (trapping) and positive selection (blast cell production) of host-I-E-reactive $V_\beta11^+$ and $V_\beta5^+$ cells but no obvious selection of $V_\beta8^+$ cells (which are not I-E reactive). Paradoxically, despite the marked enrichment for $V_\beta11^+$ and $V_\beta5^+$ cells in the blast population generated at day 3 post-transfer, the primed cells collected at 2 wk post-transfer showed a specific reduction in

$V_\beta 11^+$ and $V_\beta 5^+$ cells and a reciprocal increase in $V_\beta 8^+$ cells. $CD4^+$ cell transfer to the chimeras, by contrast, resulted in only minimal changes in the frequency of $V_\beta 11^+$ and $V_\beta 5^+$ cells (even in IFN-γ-treated hosts), despite the fact that the $CD4^+$ cells recovered at 2 wk post-transfer showed priming to host I-E (5R) antigens. Although a full interpretation of these findings will need further experimentation, two points can be made. First, the data indicate that, in addition to causing substantial trapping, Ia expression in the chimeras was clearly immunogenic for a proportion of normal parental strain $CD4^+$ cells. Second, the data on V_β ratios imply that priming in the chimeras vs. normal F_1 hosts was qualitatively different, which argues against the possibility that the chimeras contained residual BM-derived host APC.

At face value, the strong T cell priming seen in the chimeras at 2 wk post-transfer would seem to challenge the view that T cell priming is under the control of specialized BM-derived APC. The point to stress here is that, in terms of total blast cell production, proliferation of the donor $CD4^+$ cells in the chimeras was far less prominent than in normal F_1 hosts (where proliferation was presumably driven by radioresistant host APC). As suggested above, T cell contact with follicular dendritic cells (or other NBMD cells) in the chimeras was presumably overtly immunogenic for only a small proportion of the donor $CD4^+$ cells, perhaps a subset of very high affinity cells. These cells eventually underwent substantial expansion, but only after a prolonged (2 wk) period and under conditions where competition with T cells responding to antigen on typical APC was avoided. These "optimal" conditions in vivo might be difficult to reproduce in vitro. The precise specificity of the $CD4^+$ cells primed in the chimeras is still unclear. The possibility that these cells were selectively primed to tissue-specific Ia antigens seems unlikely since normal spleen cells were used to detect priming.

Since the environment of the chimeras proved to be immunogenic for normal $CD4^+$ cells (albeit a small proportion of these cells), one might expect to see comparable evidence for immunogenicity in the TG mice. Two points should be made here. First, it may be noted that for several of the TG mice tested the LN $CD4^+$ cells tended to give slightly above normal MLR to host-type APC for early (day 3) responses (Table 3, see ratios on far right). Second, it is interesting that the TG mice eventually became ill and showed lymphocytic infiltration in the liver suggestive of graft-vs.-host disease (our unpublished data). Alternatively, the pathology seen in the TG mice might have been mediated by host class I–reactive cells; the issue of tolerance/immunity at the level of $CD8^+$ cells is still under study.

The data in this paper are difficult to reconcile with the popular view that antigen presented on cells other than typical BM-derived APC is tolerogenic (25). Although this notion is well supported by in vitro experiments, evidence that antigen on "nonprofessional APC" induces tolerance in vivo is quite sparse. Indeed, given the evidence that in vitro tolerance induced by chemically fixed APC can be blocked by bystander APC (25), it is difficult to envisage how this mechanism of tolerance could operate in vivo (where bystander APC are presumably numerous). In the case of solid tissue allografts, it is well accepted that depleting grafts of "passenger leukocytes" impairs rejection (26). Such grafts rarely induce true tolerance, however, and the lack of rejection can be attributed to sequestration of the graft from the host's immune system. Perhaps the best evidence that antigen on nonprofessional APC can be tolerogenic in vivo has come from the finding that some (3, 27), though not all (28–30), transgenic lines expressing foreign Ia antigens selectively in the pancreas show tolerance at the level of $CD4^+$ cells. A puzzling feature of this model is that tolerance is evident in the thymus as well as in the periphery, a clear contrast to the TG mice reported here where neither thymus nor LN showed evidence of tolerance induction. The site and mechanism of tolerance induction in the transgenic mice is still unclear. With regard to the present data, it is of particular interest that immunity rather than tolerance occurred when the above transgenic lines were injected with normal wild-type T cells (although only when the host was irradiated or T depleted) (27). This finding does not fit easily with the notion that tolerance in the transgenic lines is simply a reflection of defective antigen presentation. However, these data from transgenic mice do agree closely with the current observation that transfer of $CD4^+$ cells to long-term chimeras led to immunity rather than tolerance.

The factors controlling tolerance induction of mature T cells are clearly still poorly understood. Recent work has suggested that the distinction between tolerance and immunity can be quite subtle. Thus, under certain conditions, T cell tolerance can be the end result of a powerful immune response (31). In light of this and the above findings, the notion that immunity and tolerance simply reflect different modes of antigen presentation will need to be re-evaluated.

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