Selective Enrichment of Major Histocompatibility Complex Class II–specific Autoreactive T Cells in the Thymic Thy0 Subset

By Ilona Kariv, Richard R. Hardy, and Kyoko Hayakawa

From the Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

Summary

We show here a unique enrichment of autoreactive T cells in the $CD4^+$ mouse thymic subset, Thy0. A single- and 10-cell AMLR (autologous mixed leukocyte reaction) assay demonstrates that more than 30% (one cell per well) and almost all (10 cells per well) Thy0 cultures from normal mice exhibit reactivity specific to autologous cells, resulting in induction of interleukin 3 secretion. In contrast, no other mature thymic or splenic $CD4^+$ T cell subsets showed such a high frequency. The majority of this AMLR reactivity in the Thy0 subset is accounted for by reactivity with self-major histocompatibility complex class II. Furthermore, antigenic selection in generating Thy0 subset is suggested by studies with T cell hybrids from a T cell receptor (TCR) Vβ transgenic mouse line, 2B4βE_H. TCR V-gene analysis of T cell hybrids revealed that those from Thy0, half of which responded to self-class II, consisted predominantly of cells that expressed endogenous TCR Vβs alone (without the transgene), unlike hybrids generated from peripheral naive T cells. Thus, we suggest that the presence of Thy0 results from selective stimulation of cells expressing TCR with sufficient affinity for autoantigens in the thymic $CD4^+$ T cell repertoire.

The thymus is the primary site for T cell development. The T cell repertoire becomes established during this intrathymic maturation process with microenvironmental interactions playing a critical role (1, 2). A variety of evidence demonstrates that binding to endogenous antigens expressed on cells in the thymus can lead to either "negative" or "positive" selection. As shown by studies with superantigen reactivities, cells with certain autoreactivities are deleted by negative selection (3–6). In contrast, binding to self-MHC molecules, either class I or II, is essential for T cells to be positively selected with maturation along the $CD8^+$ or $CD4^+$ T cell lineages, respectively (6–10).

These findings imply that the T cell repertoire is selected in the thymus on the basis of autoreactivity. However, it is not yet clear how thymocytes with autoreactive potential are selected either positively or negatively. A commonly proposed hypothesis is that the difference is due to affinity of the TCR for self-antigens: the binding affinity must be high enough to be positively selected yet low enough to avoid events leading to cell death (11, 12). The observation that the majority of peripheral T cells lack significant autoreactivity can be explained by such a TCR "affinity" selection model. However, this argument leads one to ask further about the fate of thymocytes with potentially high affinity for appropriate MHC molecules, whether they are deleted or positively selected.

In a previous paper (13), we described a unique $CD4^+$ thymic T cell subset, Thy0, in mice. This population is found in the thymus from the neonatal period on, constituting a minor subset within the $CD4^+8^-$ (heat stable antigen) $HSA^{1\ low/-}6C10^-$ mature T cell population and defined by absence of the 3G11 determinant. Cells in this fraction show rapid response to stimulation through TCR/CD3, secreting a diverse array of cytokines (13, 14). Most uniquely, this Thy0 subset shows a distinctive Vβ gene family representation that alters with age: 40–60% of Thy0 in adult BALB/c mice express TCRV β8, suggesting a significantly biased repertoire. These features contrast with the $3G11^+$ mature thymic $CD4^+$ T cell subset that we term Fr. I'. Cells in this subset produce predominantly IL-2 after stimulation and possess a distribution of Vβ families similar to peripheral $CD4^+$ T cells. These data led us to the conclusion that the majority of Fr I' cells are in the process of being exported to the periphery as naive cells, whereas Thy0 represents a secondary cell subset generated by intrathymic activation (13). According to this interpretation, Thy0 must be reactive to antigens present in the thymus. As to the nature of such self-antigens, super-

[1] *Abbreviations used in this paper:* AMLR, autologous mixed leukocyte reaction; HSA, heat stable antigen.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/93/05/1429/09 $2.00
Volume 177 May 1993 1429–1437

antigens are excluded, at least for the majority of Thy0 cells, since appropriate deletions of Vβ families associated with tolerance are seen (13).

We demonstrate here that Thy0 is indeed highly enriched for autoreactive cells, and furthermore, that the majority of these autoreactivities include the MHC class II (Ia) molecule as a part of the determinant. This conclusion is arrived at by comparing the frequency of autoreactive cells in various $CD4^+$ T cell subsets in the thymus and spleen of normal inbred mice and by establishing T cell hybrids from a $2B4\beta E_H$ TCR Vβ transgenic mouse (15). Additionally, the critical importance of antigenic selection in the generation of Thy0 is suggested by predominant usage of endogenous Vβ genes, without the transgene, in Thy0 hybrids from the β transgenic mouse. Thus, our data demonstrate that Thy0 represents a discrete subset, positively selected by sufficient affinity to autologous antigens, and remaining as a functionally active autoreactive T cell population within the thymus.

Materials and Methods

Animals. C57BL/6JN (B6), BALB/cAnN, BALB.B, and BALB.K mice were bred and maintained in our ICR animal facility. C57BL/10J (B10), B10.BR/SgSnJ, B10.D2/nSnJ, B10.A (5R)/SgSnJ, and (C57BL/10SnJ × B10.BR/SgSnJ)F_1 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B10.A mice were purchased from Animal Resources of the National Institutes of Health (Bethesda, MD). $2B4\beta E_H$ transgenic mice were kindly provided from Dr. M. Davis (Stanford University, Stanford, CA). This transgenic mouse line expressing a rearranged TCR Vβ chain gene derived from the T cell hybrid 2B4 has been described in detail (15). 2–4-mo-old female mice were used in most experiments. For the autoreactive mixed leukocyte reaction (MLR) assay, both male and female B6 mice were tested and no significant difference was seen.

Immunostaining Reagents. Hybridoma antibodies to HSA (J11d), 6C10 (SM6C10), 3G11 (SM3G11), CD4 (GK1.5), CD8 (53-6.7), CD3 (500A-A2), H-$2K^k$ (AF3-12.1), H-$2K^b$ (AF6-88.5), I-A^b (25-5-16S), I-A^k (10–3.6), I-A^d (MKD6), Vβ8 (F23.1), Vβ3 (KJ25), and their fluorescent conjugates were all prepared in our laboratory as described previously (13, 16).

Single-cell MLR and Anti-CD3 Stimulation Assays of $CD4^+$ T Cell Subsets. $CD4^+$ T cell subset definition in the thymus and spleen on the basis of HSA/3G11 or 6C10/3G11 antigen expression and cell sorting of each subset by flow cytometry was described previously (13, 16). RPMI 1641 plus 10% FCS plus 50 μM 2-ME was used as culture medium. For the single-cell MLR assay, irradiated (2,500 rad, ^{137}Cs) spleen cells from the autologous, syngeneic, or allogeneic mice were used as stimulators at 6×10^5 cells/100 μl culture medium per well against one (or 10) responder cell(s). The cell sorter was set to cloning mode and $CD4^+$ cells in the defined subsets were individually deposited into wells of a 96-well flat-bottomed plate (Costar Corp., Cambridge, MA) containing stimulator cells. To determine the background IL-3 levels, one plate was cultured with stimulator cells alone. For the anti-CD3 stimulation assay, anti-CD3 antibody (500A-A2) was coupled to 6-μ polystyrene beads (Polysciences, Inc., Warrington, PA) as described previously (17). 10^5 coupled beads in 100 μl/well were distributed onto a 96-well V-bottomed plate (Costar Corp.), and responder cells were deposited by cell sorter at one (or 10) cell(s) per well, followed by moderate centrifugation (800 rpm for 3 min) before culture.

Reactivity was examined by IL-3 release into the culture by using the IL-3–dependent cell line, DA-1, as described previously (18). To detect minute amounts of IL-3 from a single cell, we have modified the procedure used for single-cell IL-2 detection (19). In brief, after 3 d of culture, 50 μl of supernatant/well was transferred onto new plates with 100 DA-1 cells in 50 μl/well. 2 d later, the few surviving DA-1 cells were further expanded by exogenously adding activated EL-4 cell supernatant (1 μl/well) and this procedure was repeated twice at 2-d intervals. DA-1 growth level was then measured by MTT assay (20) by dissolving dye-incorporated cells with 10% SDS in 0.01 N HCl 100 μl/well at 37°C overnight. Serial dilution of EL-4 supernatant (3-d culture in an anti-CD3 coated flask) was used as a standard for IL-3 sensitivity in this bioassay. Using this method, the limit of detection was around one to two per 10^4 dilution of EL-4 supernatant and an incremental increase of OD (from 0.01 to 0.80) was seen proportional to IL-3 levels with no significant variation in triplicate culture.

Growth Factor Dependent Thy0 Cell Lines. $1–2 \times 10^5$ Thy0 cells in the thymus sorted as $HSA^-3G11^-CD4^+8^-$ were incubated with $3–6 \times 10^5$ irradiated autologous spleen cells in 100-μl culture medium in a U-bottomed plate (Costar Corp.). 2 d after incubation, rIL-2 (Amgen Biologicals, Thousand Oaks, CA) was added to allow cell proliferation. Cells were maintained by periodic stimulation with irradiated spleen cells together with EL-4 supernatant. No cell growth occurred in cultures of irradiated stimulator cells alone.

Establishment of T Cell Hybrids from $2B4\beta E_H$ Transgenic Mouse. H-2 typing of $2B4\beta E_H$ transgenic mice with B10 backgrounds (15) was carried out by examining H-2 and Ia of PBLs by an immunofluorescence staining assay. A single 3-mo-old female mouse ($H\text{-}2^{k/b}$) was killed for $CD4^+$ T cell subset analysis and cell fusion. Consistent with a previous report (15), the majority of $CD4^+$ T cells in the periphery express Vβ3, whereas $V\beta8^+$ cells are greatly reduced (<5%) compared with nontransgenic animals. Cell sorting was used to obtain 1.7×10^5 Thy0 cells from the thymus, 2.5×10^6 Fr. I cells, and 10^6 Fr. IIII cells from the spleen, which were then cultured in anti-CD3 precoated wells with irradiated autologous spleen cells. After 2 d, most cells showed visual evidence of cell activation regardless of subsets. These cultured cells were fused with a $BW\alpha^-\beta^-$ variant cell line (21) by a standard protocol and plated onto a 96-well plate. Efficiency of hybrid growth in wells with Thy0, Fr. I, and Fr. III cell fusion was 83, 70, and 100%, respectively. Cell cloning and simultaneous $CD3^+$ cell selection from each hybrid-containing well was carried out by cell sorter after immunofluorescence staining with allophycocyanin-anti-CD3. Opti-MEM (GIBCO BRL, Gaithersburg, MD) plus 4% FCS plus 50 μM 2-ME was used for maintaining T cell hybrids.

Assay for Autoreactivity in T Cell Hybrids. 10^4 T hybrid cells and 5×10^4 irradiated stimulator spleen cells were cocultured in 200 μl of hybridoma maintenance medium per well in U-bottomed 96-well plates (Costar Corp.). The plates were centrifuged (800 rpm, 3 min) before culture to promote cell contact between responder and stimulator cells. 2 d after culture, 50 μl of supernatant was tested for the presence of IL-3 by bioassay using 10^4 of DA-1 cells in 50 μl, followed by MTT assay as described previously (18). EL-4 supernatant was used as a standard.

Determination of TCR Vα and Vβ Gene Family Usage in T Cell Hybrids. Poly(A) RNA was extracted from ~10^6 cloned T hybrid cells using an mRNA isolation kit (MicroFast Track, Invitrogen Inc., San Diego, CA) according to the manufacturer's instructions. Single stranded cDNA was generated with random primers (Pharmacia LKB Biotechnology, Uppsala, Sweden) using M-MLV re-

verse transcriptase (GIBCO BRL) at 42°C for 1 h. PCR was carried out in 50 μl with 1.25 U Taq DNA polymerase (Promega Corp., Madison, WI). The amplification protocol consisted of five cycles of 95, 62, and 72°C (30 s at each temperature), followed by 30 cycles of 95, 58, and 72°C (30 s at each temperature). Oligonucleotide sequences of the specific primers for determination of TCR Vα and Vβ gene families were previously described by Casanova et al. (22). To identify the Vβ8.1/Vβ8.2 TCR gene family together, a mixed oligo CATTCTGGAGTTGGCT$^{T}/_{A}$CCC was used (Dr. A. Caton, Wistar Institute, Philadelphia, PA, personal communication). The reaction products were separated by electrophoresis in a 2% agarose gel and visualized by UV light after staining with ethidium bromide. The intensity of bands with β-actin and VαBWB (derived from the BWα$^-$β$^-$ cell line; 23) provided controls for the presence of mRNA presence in the T cell hybrid samples. The specificity of the primers was tested by using several T cell hybrids with known TCR V-gene usage (24) (provided by Dr. P. Nakajima at this Institute) and also by comparing results with all panels of T cell hybrids obtained here.

Results

A High Frequency of Autoreactive T Cells in the Thy0 Subset Is Revealed by Single Cell Autologous Mixed Leukocyte Reaction (AMLR) Analysis. To determine the frequency of autoreactive T cells, we have utilized a single-cell MLR assay. Single cells from the Thy0 subset were cloned into individual wells by cell sorter and irradiated spleen cells were used as stimulators. The response was measured by secretion of IL-3, which is a generic cytokine for any type of activated CD4$^+$ T cell (25). As Fig. 1 shows, around 30–40% of Thy0 cells from C57BL/6 mice (B6) were responsive to autologous cells (B6 anti-B6) above background level. This IL-3 secretion is induced by specific autologous cell reactivity since the frequency of alloreactive cells (B6 anti-BALB/c) was significantly lower (Fig. 1) and IL-3 secretion was not observed in the absence of stimulator cells. The presence of a T cell in each well was verified by the fact that 97% of wells showed IL-3 secretion when stimulated with anti-CD3 as summarized in Table 1.

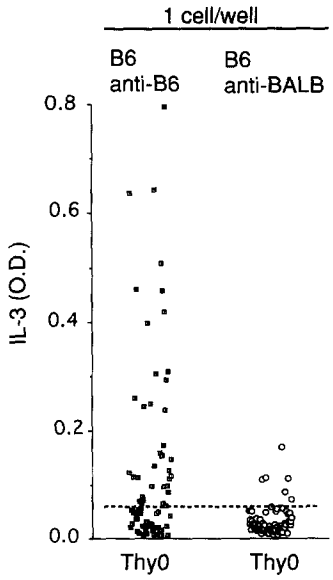

Figure 1. A high frequency of autoreactive cells in the Thy0 subset is detected using a single-cell MLR assay. Thy0 cells from a B6 mouse were incubated with irradiated spleen cells either from B6 (autologous) or BALB/c mice at 1 Thy0 responder cell/well × 96 wells. Induction of IL-3 secretion in the MLR culture was determined by bioassay. Background IL-3 levels in cultures of stimulator cells alone yielded an OD of 0.01 ± 0.02 (mean ± SD, n = 96), resulting in a cutoff point of 0.05 (mean + 2 SD) (---). Data from B6 anti-B6 MLR culture presented here included 36 positive wells with a mean OD of 0.23 ± 0.02, and 56 negative wells with a mean OD of 0.01 ± 0.02. Six wells were scored as positive in the B6 anti-BALB MLR. Data from two experiments yielded a similar frequency of positive wells.

Table 1. *A High Frequency of Autoreactive T Cells in Thy0 Detectable by MLR Assay*

		IL-3$^+$ wells			
		1 cell/well		10 cells/well	
CD4$^+$ subsets		AMLR	anti-CD3	AMLR	anti-CD3
		%			
Thymus	Thy0	<1	100 37	97	96
Thymus	Fr. I′	<1	95	2	100
Spleen	Fr. I	ND	ND	1	100
Spleen	Fr. III	ND	ND	8	100

96 wells (either 1 or 10 cells/well) were cultured per each sample group. Positive for IL-3 presence (IL-3$^+$) was scored as wells showing >0.05 OD level. By anti-CD3 stimulation, most samples yielded OD ~1.0 irrespective of responder cell numbers.

Enrichment of Autoreactive T Cells Is Unique to the Thy0 Subset. To determine whether this high frequency of autoreactive cells in the MLR system was unique to Thy0 or instead a feature of any secondary subset that is naturally generated, we compared thymic Fr. I′ and Thy0 with splenic Fr. I and Fr. III. The gates for each fraction are shown in Fig. 2. As we have described previously (13, 16, 26), six mature subsets can be identified among CD4$^+$ T cells: two in thymus (Fr. I′ and Thy0, defined within the HSA$^{low/-}$6C10$^-$ fraction by 3G11 expression; 13) and four in the spleen (Fr. I–IV, defined by 6C10 and 3G11 expression; 16) (Fig. 2).

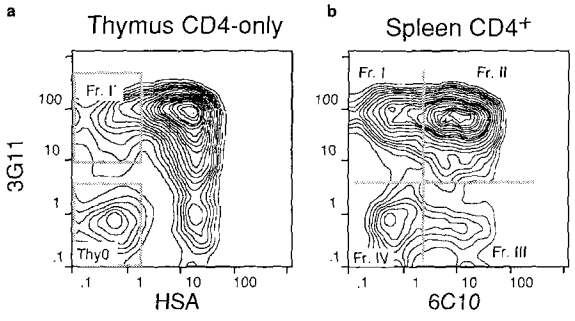

Figure 2. CD4$^+$ T cell subsets in the thymus and spleen of B6 mice. (*a*) Thymocytes in a B6 mouse (3-mo-old) were simultaneously stained with APC-anti-CD4, PE-anti-CD8, FL-anti-HSA (J11d), and biotin-SM3G11 plus TR-avidin. CD4$^+$8$^-$ cells are gated for this display. Regions for the HSA$^-$ mature T cell subsets used for experiments in this study, Fr I′ and Thy0 cell subsets, are indicated. (*b*) Spleen cells were stained with APC-anti-CD4, FL-SM6C10, and biotin-SM3G11 plus TR-avidin. Four CD4$^+$ T cell subsets (Fr. I–IV) are defined by 6C10 and 3G11 levels.

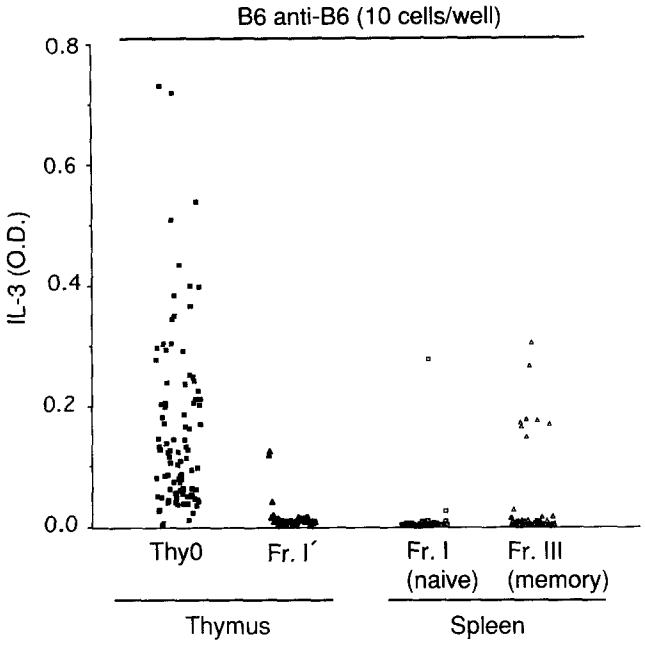

Figure 3. A high incidence of AMLR reactive cells is unique to the Thy0 subset. Cells in the two thymic CD4+ T cell subsets (Thy0 and Fr. I') and two splenic CD4+ T cell subsets (Fr. I and Fr. III) of a B6 mouse were analyzed by AMLR assay using 10 responder cells/well × 96 wells per subset. 96% of Thy0 wells produce IL-3. In three experiments, Thy0 cells consistently showed >95% response under this condition.

Among these, Fr. III exhibits the characteristics of a secondary population, secreting a diverse array of cytokines upon stimulation (16, 26), similar to the thymic Thy0 subset (13, 14). In contrast, Fr. I shows exclusive IL-2 secretion and likely represents the "naive" subset most recently exported from the thymus (16, 26, 27).

To increase the sample size for our comparison, 10 cells were deposited per well. IL-3 should be detected if, at minimum, one responding cell is present per well as suggested from single-cell culture results. As shown by data presented in Fig. 3 and Table 1, the majority (>90%) of Thy0 wells exhibited IL-3 secretion in AMLR as expected, whereas only 1–2% of wells from thymic Fr. I' and splenic Fr. I responded in this system. Another naive T cell subset, Fr. II, also showed a low frequency similar to Fr. I (data not shown). Although some increase (8%) was observed in Fr. III wells, the difference between Thy0 and Fr. III is clear with regard to the extent of enrichment of AMLR responding cells. Data from anti-CD3 stimulation confirms the presence of functional T cells capable of secreting IL-3 in all wells tested (Table 1).

The Majority of Autoreactive Thy0 Cells Show Specificity to MHC Class II+ Cells. After activation of Thy0 cells by AMLR, autoreactive Thy0 cells could be maintained in culture by periodic restimulation with irradiated syngeneic spleen cells together with growth factor addition. After restimulation of cells, activated cells show increase of cell size and internal cell granularity as demonstrated by forward and right angle scatter measurements by FACS® analysis (Becton Dickinson & Co., Mountain View, CA) (Fig. 4). We found that activation of such Thy0 bulk cell cultures by irradiated syngeneic spleen cells is dependent on either B or non B/T cells in the spleen (Fig. 4, *top*), but not T-lineage cells (Fig. 4, *bottom*). The syngeneic specificity was demonstrated by mutually exclusive responsiveness with C57BL/6- and BALB/c-derived Thy0 cell lines. Furthermore, specificity for class II molecules was suggested by the observation that these responses were inhibited by anti-Ia antibodies in a haplotype-specific fashion (Table 2). The fact that almost all cells in bulk cultures responded to B but not T cells, as shown by FACS® analysis, suggests that, at a minimum, a majority exhibit similar specificities with apparent involvement of MHC class II.

A High Frequency of Specific Autoreactive T Cell Hybrids in the Thy0 Subset from a 2B4βE$_H$ Transgenic Mouse. We then attempted to see whether the differential responsiveness in AMLR is due to T cell specificity and not to non-TCR-mediated effects. If the reactivity is principally determined by specificity, then it is likely that TCR V gene usage will differ

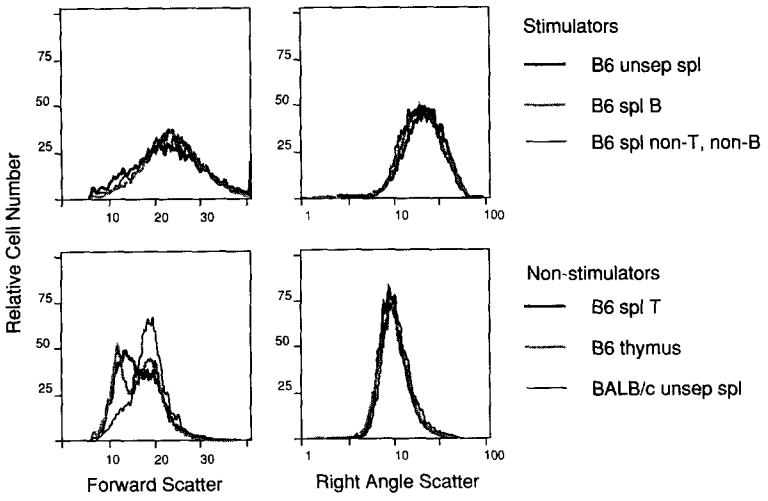

Figure 4. Class II+ cells are largely responsible for inducing AMLR activity in Thy0. 2 wk after initiation of B6 anti-B6 Thy0 cell activation and cell expansion, a maintained cell line (8074a) was resorted based on cell size difference by forward light scatter to eliminate residual stimulator spleen cells in the culture. As fresh stimulator cells, B6 spleen cells were stained with PE-anti-B220 (RA3-6B2) and APC-anti-CD5 (53-7). B, T, and non-B/T cells were then sorted as B220+CD5−, B220−CD5+, and B220−CD5− fractions, respectively. B6 thymocytes, B6 spleen cell fractions, and BALB/c spleen cells were irradiated before use. 2 × 10^5 responder 8074a cells were incubated with 5 × 10^5 stimulator cells together with rIL-2 in a microtiter plate. 3 d after incubation, cells were analyzed by FACS® for the forward and right angle scatter measurements. Most irradiated stimulator cells died and were eliminated by computer gating from these measurements as propidium iodide positive cells.

Table 2. *Anti-Ia Antibody Inhibits Induction of Thy0 Cell Activation in AMLR*

Thy0 Culture line	Reactivity	Inhibitor	Δ Increase of activated cells	Percent inhibition
807-4a	B6 anti-B6	(−)	1,066	–
		anti-I-A^b	248	77
		anti-I-A^d	980	8
820	BALB anti-BALB	(−)	1,242	–
		anti-I-A^b	1,634	0
		anti-I-A^d	0	100

807-4a and 820 cell lines are derived from the Thy0 subset in B6 and BALB/c mice, respectively. A portion of early expanded cell cultures was rested for 10 d and restimulated with irradiated appropriate spleen cells with or without anti-I-A antibodies. Approximately 10^5 responder cells and 2×10^5 stimulator cells were cultured in a U-bottomed plate. Anti-I-A^b (25-5-16S) and anti-I-A^d (MKD6) antibodies were used at 50 μg/ml where toxic effect to B cells is 0–10%. 3 d after culture, cells were harvested for measurement of live cell numbers and cell size distribution by cell sorter. The number of activated cells (Δ increase) was obtained from the percentage of large cells (arbitrarily gated by forward scatter) within total live cells, counted by cell sorter, followed by subtraction of such numbers obtained from the culture without stimulator (Δ increase). The numbers presented are a mean of duplicate culture where deviation was <10% in all groups.

between Thy0 and the peripheral naive T cell populations. We have used the TCR Vβ single transgenic mouse line (2B4βE$_H$) for this purpose. This TCR Vβ gene (of the Vβ3 gene family) from a 2B4 T cell hybrid encodes a receptor specific for pigeon cytochrome *c* in the context of I-E^k when combined with the appropriate TCR Vα (15, 24). Further, a majority of peripheral CD4$^+$ T cells from mice with the H-2^k haplotype show exclusive Vβ3 transgene expression (15). Thus, we anticipated that this restriction of Vβ would amplify the difference in TCR gene usage between Thy0 and the peripheral naive T cell populations.

We found that in the 2B4βE$_H$ transgenic mice of the H-$2^{k/b}$ background (B10.BR/B10), the Thy0 subset in the thymus and the four peripheral subsets (Fr. I–IV) are at normal levels, possessing cytokine profiles and cell surface phenotypes similar to those in normal (B10 × B10.BR)F_1 mice (data not shown). We have used one such H-$2^{k/b}$ 2B4βE$_H$ mouse, generating T cell hybrids from the Thy0, Fr. I, and Fr. III subsets simultaneously. As summarized in Table 3, 10–20 TCR/CD3$^+$ T cell hybrid clones were randomly established from each subset. All have the potential to secrete IL-3 as elicited by anti-CD3 stimulation with no significant difference between subsets (data not shown). Potential autoreactivity was assayed by measuring the induction of IL-3 after incubation with irradiated spleen cells from (B10 × B10.BR)F_1, B10, or B10.BR mice. To distinguish a self-MHC–related specific response from activation by unknown cell interactions unrelated to self-MHC, B10.D2 spleen cells were also

Table 3. *A High Frequency of Autoreactive T Hybrids from the Thy0 Subset of a 2B4βE$_H$ (H-$2^{k/b}$) Mouse*

2B4βE$_H$ CD4$^+$ T cell subsets	No. of CD3$^+$ hybrids	No. of autoreactive hybrids					Self-MHC specific hybrids
		F_1 alone	F_1/B10	F_1/B10.BR	unclassified	N/A	
Thymus							
Thy0	12	0	6	1	0	3*	7/12
Spleen							
Fr.I	13	0	0	0	3	4	0/13
Fr.III	22	0	0	0	4	4	0/22

T cell hybrid clones were established from the CD4$^+$ T cell subsets of a 2B4βE$_H$ transgenic mouse (B10.BR/B10). All hybrids expressing TCR/CD3 on the cell surface were tested for autoreactivity in the MLR assay. Hybrids showing induction of IL-3 secretion by incubation with (B10 × B10.BR)F_1 stimulator spleen cells are listed as autoreactive hybrids. The specific reactivity in relation to self-MHC was determined by comparing responses to F_1, B10, B10.BR, and B10.D2 stimulator cells. F_1/B10 (or B10.BR) respond to both F_1 and B10 (or B10.BR) stimulators. The hybridomas showing induction of IL-3 secretion regardless of mouse strains are listed as unclassified hybrids. All hybrids listed here show IL-3 secretion by anti-CD3 stimulation (OD 0.4–0.9) and AMLR responding hybrids show OD 0.2–0.8 (OD 0.00–0.05 without stimulation).
* Not applicable due to constitutive IL-3 secretion.

tested as stimulators. As data reveal in Table 3, over half (7 of 12) of Thy0-derived hybrids show specific autoreactivity. Most of these are specific to both (B10 × B10.BR)F_1 and B10. None of the hybrids from other subsets show such MHC-associated self-reactivity except for an apparent nonspecific response ("unclassified").

Autoreactivity in Thy0 Hybrids Is MHC Class II Specific. The specificity to MHC class II was further demonstrated by differential reactivity to stimulators and by inhibition with anti-Ia mAbs. As Table 4 shows, out of seven Thy0 autoreactive hybrids (with H-$2^{k/b}$), six show reactivity to spleen cells from mice with $K^bI\text{-}A^b$ haplotype, B10, B10.A (5R), and BALB.B, in addition to H-$2^{k/b}$ F_1 mouse. Another hybrid shows reactivity to cells with $K^kI\text{-}A^kI\text{-}E^k$. Concordantly, responses by these six T hybrids to H-2^b stimulator cells were specifically inhibited by anti-I-A^b antibody as represented in Table 5 (50A5, 50G1). In contrast, nonspecific IL-3 induction unrelated to MHC (designated as unclassified) as seen with some Fr. III-derived hybrids (48D3, 48C2) was not inhibitable by anti-I-A.

TCR Vα and Vβ Gene Family Representation by 2B4βE$_H$ Thy0 Hybrids: Thy0 Is Generated by Antigen-specific Recruitment. We then examined the diversity of TCR genes used in these Thy0 hybrids (Table 6). This was determined by reverse transcriptase-PCR (RT-PCR) of mRNA using V- gene family–specific 5′ oligos, which allows rapid determination of TCR Vα and Vβ gene family expression (22). As recently reported (22, 28), the majority of T hybrids show expression of both TCR Vα alleles which helps in demonstrating that these Thy0 hybrids from a single mouse are all clonally unrelated, utilizing diverse Vα genes. Distinctively, we found that a large fraction of hybrids (9/12, 75%) do not express the Vβ3 transgene. This contrasts with data from hybrids of splenic naive cells (Fr. I) where the majority of hybrids (11/13, 84%) retain expression of the transgene as confirmed by anti-Vβ3 surface staining, consistent with a previous report with nontransformed cells (15). Our data further reveal

Table 4. *Autoreactive Thy0 Cell Hybrids Are Specific to MHC Class II*

	H-2				Thy0 hybrid reactivity pattern	
Stimulator	K	A	E	D	F_1/B10	F_1/B10.BR
B10	b	b	–	d	+	–
B10.BR	k	k	k	k	–	+
B10.D2	d	d	d	d	–	–
B10.A (5R)	b	b	b/k*	d	+	–
B10.A	k	k	k	d	–	+
BALB.B	b	b	–	b	+	–
BALB.K	k	k	k	k	–	+
BALB/c	d	d	d	d	–	–

Six Thy0 hybrids showing F_1/B10 reactivity and one Thy0 hybrid with F_1/B10.BR reactivity were tested by MLR assay. All Thy0 hybrids of the F_1/B10 group showed a positive response to mice with H-$2K^bI\text{-}A^b$.
* $E\beta^bE\alpha^k$ ($E^{b/k}$) in contrast with $E\beta^kE\alpha^k$ (E^k).

Table 5. *Specific Inhibition of Thy0 Response in AMLR by Anti-I-A Antibody*

		Responder			
		MHC specific Thy0 hybrids		Nonspecific Fr III hybrids	
Stimulator	Inhibitor	50A5	50G1	48D3	48C2
		OD			
B10 spleen	None	0.389	0.766	0.394*	0.485
	Anti-I-A^b	0.012	0.035	0.315	0.484
	Anti-I-A^d	0.353	0.735	0.424	0.377
(–)	None	0.001	0.004	0.022	0.338

Anti-I-A^b and anti-I-A^d hybridoma antibodies as inhibitors were described in Table 2. Data from two Thy0 hybrids (50A5, 50G1) showing I-A^b specificities are shown as representative.
* Among samples of nonspecific IL-3 release, 48D3 was grouped as "unclassified" and 48C2 was grouped as not applicable in the Fr. III hybrids in Table 3.

Table 6. *Clonal Independence and Predominant Endogeneous Vβ Gene Usage in Thy0 Hybrids*

Thy0 hybrids	Reactivity to self-Ia	Transcribed TCR V-gene families	
		Vα	Vβ
50A5	+(b)	6, 11	**3***
50D4	+(b)	4, 8	**3**
50F2	+(b)	8	**3, 8.1-.2**
50C5	+(b)	2, 13.1	1
50D1	+(b)	1, 3	1
50G1	+(b)	5, 34S-281	1, **8.1-.2**
50A2	+(k)	4	14, 16
50B5	–	ND	8.3
50G2	–	10, 12	**8.3**
50B1	Unknown	3, 13.1	**8.1-.2**
50E4	Unknown	4, 34S-281	**8.1-.2**
50F1	Unknown	8, 34S-281	1

TCR V-gene families were determined by RT-PCR where Vα and Vβ gene family assignment was as described previously (22). Three hybrids (B1, E4, F1) are constitutive IL-3 producers (N/A in Table 3).
* Numbers in bold: cell surface expression was confirmed by fluorescence staining analysis using anti-Vβ3 (KJ25) or anti-Vβ8 (F23.1) antibody.

that among I-A^b–specific autoreactive hybrids, one half (3/6) utilize exclusively nontransgene Vβ genes in encoding this self-reactivity. One hybrid (A50F2) coexpresses Vβ3 and Vβ8 on the cell surface. These data, clonal independence and bias toward endogenous Vβ gene expression, strongly suggest that Thy0 cells are generated by recruitment of cells by antigenic selection mediated by TCR/CD3. Whereby, expression of appropriate (self)-specificities is allowed by expressing endogenous Vβ genes preferentially to this transgene.

Discussion

The study presented here demonstrates an enrichment of MHC-specific autoreactive cells in the Thy0 subset, unlike any other $CD4^+$ T cell subset. Single-cell AMLR analysis reveals that >30% of Thy0 cells are responsive to autologous or syngeneic spleen cells, being induced to secrete cytokine(s) as demonstrated by an IL-3 assay. In contrast, this frequency in naive T cell populations in the thymus and spleen is much lower, 0.1 (−1.0)%, where only 1–2 wells out of 96 showed any response even when 10 responders were plated per well. This difference in frequency is also demonstrated by comparing hybrid cell lines generated from $CD4^+$ cell subsets of the 2B4βE_H transgenic mouse line. Our data reveal that more than half of randomly chosen Thy0 cell hybrids show MHC-specific autoreactivity, whereas hybrids from other $CD4^+$ T cell subsets do not. Since all T hybrids were made after initial activation in culture, these data exclude possible differences in cell cycle between subsets that might have biased results in our MLR assay. Thus, considering both the limitation of the single-cell MLR assay with normal cells (in terms of sensitivity) and the potential diversity of antigens (which might be absent from our stimulator preparation), it appears reasonable to conclude that the majority of Thy0 cells are reactive with autologous cells and that this feature distinguishes the Thy0 subset from all other $CD4^+$ T cell subsets.

Our data show that most Thy0 autoreactivity appears to be MHC class II (Ia) specific. Thy0 cells respond to Ia-expressing cells in an Ia-specific manner, and the response is specifically inhibited by monoclonal anti-Ia antibody. Although it is not clear whether the response is to class II itself or to self-peptides/class II as reported recently (29, 30), our data nevertheless imply that autoantigen recognition by Thy0 involves the MHC class II molecule in a fashion similar to foreign antigen recognition by $CD4^+$ T cells. These findings raised the issue of how such autoreactive Thy0 cells in the thymus relate to peripheral naive T cells. A critical question was whether the majority of Thy0 cells could convert into nonautoreactive T cells in the periphery with TCR expression unchanged (11). Alteration of cell surface molecule expression associated with the progression of thymic T cells to peripheral T cells might change signal transduction pathways, resulting in differential reactivity as reflected in the difference between naive and memory T cells (31, 32).

An alternative is that affinity to the autoantigens mediated by TCR/CD3 is crucial and determines the fate of $CD4^+$ T cells in the thymus, one for the naive T cell pool and another for the Thy0 subset. In this case, the T cell repertoire would likely differ between Thy0 and the peripheral naive T cell populations. This prediction is supported by our data with 2B4 TCR Vβ transgenic mice, with the advantage of exclusive transgene expression in such mice (15). In this transgenic mouse, transgene Vβ randomly combines with endogenous Vα, allowing some flexibility in T cell repertoire with limited Vβ usage. Thus, if Thy0 cells are selected by antigen specificity different from naive cells, then transgene expression will positively or negatively affect the generation of this subset.

Initially, we found that the frequency of Thy0 is greatly reduced in 2B4βE_H transgenic mice on an H-$2^{k/k}$ background, whereas the frequencies of thymic Fr. I′ and peripheral subsets are unaffected (27). This supports the notion that Thy0 is specifically affected because of a limitation of available (autoreactive) repertoire. Second, as shown here by analysis of T hybrids, within the Thy0 subset that is generated, nontransgene-expressing cells predominate, in contrast with results from naive T cell subsets. Our result does not exclude the possibility of selective loss of transgene in the process of establishing the cell lines. However, the fact that >50% of Thy0 hybrids show class II–specific autoreactivity (similar to our results with normal inbred mice) and that one half of these autoreactive hybrids lack transgene expression, strongly supports the idea that endogenous genes were indeed utilized for expressing autoreactivity in situ regardless of transgene expression. Thus, our data indicate that Thy0 generation is dependent on the specificity that the T cell expresses. Finally, Thy0 hybrids are all clonally independent and the Thy0 frequency increases with age, suggesting that Thy0 cells accumulate by repeated recruitment of numerous individual cells with a minimum of clonal expansion.

These data with 2B4 TCR transgenic mice allowed us to solve a question raised in our previous study (13). Although a distinct repertoire for Thy0 was suggested by data with BALB/c mice, distinct TCR gene family representation was not evident in Thy0 in young mice and in several strains, including C57BL/6 (13) studied here. From the results obtained here, we suggest that antigens and/or TCR genes involved in such self-specificities are diverse enough to obscure differences in TCR Vβ gene family representation. It would be interesting to determine whether the gradual increase of V$\beta 8^+$ cells with ontogeny clearly found in Thy0 of BALB/c mice is due to a preferential selection of cells with a more restricted reactivity or instead to a clonal expansion promoted by BALB/c background genes.

Taken together, our data demonstrate that Thy0 is a unique subset generated by a positive response to endogenous antigens (positively selected) expressed on the intact cell surface. Previous reports demonstrating a response by thymocytes to autologous or syngeneic spleen cells (33) may largely reflect the activity of Thy0. The antigenic determinants appear to be predominantly self-MHC class II and our preliminary results show that Thy0 cell lines also react with a stromal cell–enriched class II^+ thymic cell preparation, suggesting that thymic stromal cells are involved in generating Thy0. However, it is not clear which type of class II^+ cells,

whether cortical or medullar epithelial cells, dendritic cells, or macrophages are concerned (9, 34). Since the Thy0 subset in adult mice shows appropriate deletion of $V\beta^+$ T cells related with tolerance (13), this suggests that the Thy0 precursor population is generated similarly to other $CD4^+$ T cells based on affinity to class II. However, the generation of Thy0 may require both TCR with substantially higher affinity for available autoantigens/class II and functional maturity to be activated by antigen binding. Thus, this selection process may only occur at a later stage in the cells recognized as $HSA^{low/-}6C10^-$ after $CD4^+$ T cell generation (13, 35, 36), and class II^+ cells must be accessible to such cells.

The presence of autoreactive T cells and the ability to generate autoreactive T cell lines has been documented largely with the peripheral lymphoid system of normal and autoimmune animals (11, 12, 37–40). Their MHC class II specificity, similar to Thy0, is well established as a common feature of such peripheral autoreactive T cell lines (11, 12, 38, 39, 41). Since these autoreactive T cell lines are frequently obtained from mice in the course of immunization, the question has been raised previously whether such autoreactive cells constitute a significant portion of normal T cell repertoire, or alternatively, whether their reactivity resulted from activation of T cells followed by successive in vitro stimulation (12, 41). Our data demonstrates that functional MHC-specific autoreactive T cells are very rare in the periphery of normal mice, and thus suggest that the majority of Thy0 may not be exported to the periphery, or if exported, that such cells become anergic or die because of the continual presence of stimulating antigen (42) as a mechanism for tolerance. Therefore, the concept of an abundance of autoreactive T cells appears unlikely. Rather, our study here raises an alternate possibility that peripheral autoreactive T cells may arise from Thy0 whose normal fate (anergy or death) is disregulated by immunization or the disease state. In this regard, understanding the biological significance of Thy0 cell with autoaggressive potential is important for future studies.

We thank Ms. Betsy T. Lin for preparation of reagents and providing expert technical help throughout these studies. Ms. Susan A. Shinton helped in the operation of the cell sorter. Dr. P. Nakajima provided the DA-1 cell line and T cell hybrids for standards and, in addition, valuable help in our cell fusion experiment. We are indebted to Dr. M. Davis for providing transgenic mice and to Dr. A. Caton for helpful discussion and information in the assignment of TCR V genes. We also acknowledge Drs. M. Bosma, M. Bookman, and P. Nakajima for critical reading of this manuscript.

This study was supported by grants from the National Institutes of Health (CA-06927 and RR-05539).

Address correspondence to Dr. Kyoko Hayakawa, Institute for Cancer Research, Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, PA 19111.

Received for publication 13 November 1992 and in revised form 15 January 1993.

References

1. Bevan, M. 1977. In a radiation chimera host H-2 antigens determine the immune responsiveness of donor cytotoxic cells. *Nature (Lond.).* 269:417.
2. Zinkernagel, R., G. Callahan, A.C. Althage, S.P. Klein, and J. Klein. 1978. On the thymus in the differentiation of H-2 self-recognition by T cells: evidence for dual recognition? *J. Exp. Med.* 147:882.
3. Kappler, J.W., N. Roehn, and P. Marrack. 1987. T cell tolerance by clonal elimination in the thymus. *Cell.* 49:273.
4. MacDonald, H.R., R. Schneider, R.K. Lees, R.C. Howe, H. Acha-Orbea, H. Festenstein, R.M. Zinkernagel, and H. Hengartner. 1988. T-cell receptor $V\beta$ use predicts reactivity and tolerance to Mls^a-encoded antigens. *Nature (Lond.).* 332:40.
5. Kisielow, P., H. Bluthmann, U.D. Staerz, H. Steinmetz, and H. von Boehmer. 1988. Tolerance in T cell receptor transgenic mice involves deletion of non-mature $CD4^+8^+$ thymocytes. *Nature (Lond.).* 333:742.
6. Sha, W.C., C.A. Nelson, R.D. Newberry, D.M. Kranz, J.H. Russell, and D.Y. Loh. 1988. Positive and negative selection of an antigen receptor on T cells in transgenic mice. *Nature (Lond.).* 336:73.
7. Teh, H.S., P. Kisielow, B. Scott, H. Kishi, Y. Uematsu, H. Bluthmann, and H. von Boehmer. 1988. Thymic major histocompatibility complex antigens and the $\alpha\beta$ T-cell receptor determine the CD4/CD8 phenotype of T cells. *Nature (Lond.).* 335:229.
8. Kaye, J., M.-L. Hsu, M.-E. Sauron, S.C. Jameson, N.R.J. Gascoigne, and S.M. Hedrick. 1989. Selective development of $CD4^+$ T cells in transgenic mice expressing a class II MHC-restricted antigen receptor. *Nature (Lond.).* 341:746.
9. Benoist, C., and D. Mathis. 1989. Positive selection of the T cell repertoire: where and when does it occur? *Cell.* 58:1027.
10. Zuniga-Pflucker, J.C., D.L. Long, and A.M. Kruisbeek. 1989. Positive selection of $CD4^-CD8^+$ T cells in the thymus of normal mice. *Nature (Lond.).* 338:76.
11. Glimcher, L.H., D.L. Longo, I. Green, and R.H. Schwartz. 1981. Murine syngeneic mixed lymphocyte response. I. Target antigens are self Ia molecules. *J. Exp. Med.* 154:1652.
12. Zauderer, M. 1989. Origin and significance of autoreactive T cells. *Adv. Immunol.* 45:417.

13. Hayakawa, K., B.T. Lin, and R.R. Hardy. 1992. Murine thymic CD4+ T cell subsets: a subset (Thy0) which secretes diverse cytokines and over-expresses the Vβ8 T cell receptor gene family. *J. Exp. Med.* 176:269.
14. Bendalac, A., and R.H. Schwartz. 1991. CD4+ and CD8+ T cells acquire specific lymphokine secretion potentials during thymic maturation. *Nature (Lond.).* 353:68.
15. Berg, L.J., A.M. Pullen, B. Fazekas de Dt. Groth, D. Mathis, C. Benoist, and M.M. Davis. 1989. Antigen/MHC–specific T cells are preferentially exported from the thymus in the presence of their MHC ligand. *Cell.* 58:1035.
16. Hayakawa, K., and R.R. Hardy. 1988. Murine CD4+ T cell subsets defined. *J. Exp. Med.* 168:1825.
17. Taplits, M.S., P.A. Henkart, and R.J. Hodes. 19898. T helper cytoplasmic granules. Exocytosis in response to activation via the T cell receptor. *J. Immunol.* 141:1.
18. Haberman, A.M., C. Moller, D. McCreedy, and W.U. Gerhard. 1990. A large degree of functional diversity exists among helper T cells specific for the same antigenic site of influenza hemagglutinin. *J. Immunol.* 145:3087.
19. Rocha, B., and A. Bandeira. 1988. Limiting dilution of analysis of interleukin 2-producing mature T cells. *Scand. J. Immunol.* 27:47.
20. Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods.* 65:55.
21. White, J., M. Blackman, J. Bill, J. Kappler, P. Marrack, D.P. Gold, and W. Born. 1989. Two better cell lines for making hybridomas expressing specific T cell receptors. *J. Immunol.* 143:1822.
22. Casanova, J.-L., P. Romero, C. Widmann, P. Kourilsky, and J.L. Maryanski. 1991. T cell receptor genes in a series of class I major histocompatibility complex–restricted cytotoxic T lymphocyte clones specific for a *Plasmodium berghei* nonapeptide: implications for T cell allelic exclusion and antigen-specific repertoire. *J. Exp. Med.* 174:1371.
23. Letourneur, F., and B. Malissen. 1989. Derivation of T cell hybridoma variant deprived of functional T cell receptor α and and β chain transcripts reveals a nonfunctional α-mRNA of BW5147 origin. *Eur. J. Immunol.* 19:2269.
24. Winoto, A., J.L. Urban, N.C. Lan, J. Goverman, L. Hood, and D. Hansburg. 1986. Predominant use of a V_α gene segment in mouse T-cell receptors for cytochrome c. *Nature (Lond.).* 324:679.
25. Mosmann, T.R., H. Cherwinski, M.W. Bond, M.A. Giedlin, and R.L. Coffman. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* 136:2348.
26. Hayakawa, K., and R.R Hardy. 1991. Murine CD4+ T cell subsets. *Immunol. Rev.* 123:145.
27. Hayakawa, K., B.T. Lin, and R.R. Hardy. 1992. Murine CD4+ T cell subsets generated by antigen independent and dependent mechanisms. *Chem. Immunol.* 54:103.
28. Malissen, M., J. Trucy, E. Jouvin-Marche, P.-A. Cazenave, R. Scollay, and B. Malissen. 1992. Regulation of TCR α and β gene allergic exclusion during T-cell development. *Immunol. Today.* 315.
29. Rudensky, A.Y., P. Preston-Hurlburt, S.-C. Hong, A. Barlow, and C.A. Janeway, Jr. 1991. Sequence analysis of peptides bound to MHC class II molecules. *Nature (Lond.).* 353:622.
30. Lorenz, R.G., and P.M. Allen. 1988. Direct evidence for functional self-protein/Ia-molecule complexes *in vivo*. *Proc. Natl. Acad. Sci. USA.* 85:5220.
31. Cerottini, J.-C., and H.R. MacDonald. 1989. The cellular basis of T-cell memory. *Annu. Rev. Immunol.* 7:77.
32. Matsui, K., J.J. Boniface, P.A. Reay, H. Schild, B. Fazekas de St. Groth, and M.M. Davis. 1991. Low affinity interaction of peptide–MHC complexes with T cell receptors. *Science (Wash. DC).* 254:1788.
33. Von Boehmer, H., and W.J. Byrd. 1972. Responsiveness of thymus cells to syngeneic and allogeneic lymphoid cells. *Nature (Lond.).* 235:50.
34. Van Ewijk, W. 1991. T-cell differentiation is influenced by thymic microenvironments. *Annu. Rev. Immunol.* 9:591.
35. Crispe, N., and M.J. Bevan. 1987. Expression and functional significance of the J11d marker on mouse thymocytes. *J. Immunol.* 138:2013.
36. Wilson, A., L.M. Day, R. Scollay, and K. Shortman. 1988. Subpopulations of mature murine thymocytes: properties of CD4−CD8+ and CD4+CD8− thymocytes lacking the heat-stable antigen. *Cell. Immunol.* 117:312.
37. Weksler, M.E., C.E. Moody, Jr., and R.W. Kozak. 1981. The autologous mixed-lymphocyte reaction. *Adv. Immunol.* 31:271.
38. Glimcher, L.H., and E.M. Shevach. 1982. Production of autoreactive I region-restricted T cell hybridomas. *J. Exp. Med.* 156:640.
39. Finnegan, A., B.W. Needleman, and R.J. Hodes. 1990. Function of autoreactive T cells in immune responses. *Immunol. Rev.* 116:15.
40. Strober, W., and S.P. James. 1990. Immunoregulatory function of human autoreactive T-cell lines and clones. *Immunol. Rev.* 116:117.
41. Faherty, D.A., D.R. Johnson, and M. Zauderer. 1985. Origin and specificity of autoreactive T cells in antigen-induced populations. *J. Exp. Med.* 161:1293.
42. Ramsdell, F., and B.J. Fowlkes 1992. Maintenance of *in vivo* tolerance by persistence of antigen. *Science (Wash. DC).* 257:1130.