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MACROPHAGES PHAGOCYTOSE THYMIC LYMPHOCYTES WITH PRODUCTIVELY REARRANGED T CELL RECEPTOR α AND β GENES

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The thymus is critical for the production of mature, recirculating T cells. Congenitally athymic mammals and neonatally thymectomized mice, have profound deficits in T cell numbers and immunologic functions. Such athymic individuals do not reject skin grafts nor do they resist a variety of infectious agents (1–5), and few lymphocytes are noted in the thymus-dependent areas of lymphoid tissues (5–7). The thymus is also the main site for rearranging TCR-α and -β genes (8–12). The repertoire of α/β receptors is shaped by deleting cells that react directly with self MHC (13) and by selecting cells that recognize foreign antigens in association with self MHC (14, 15). These negative and positive selections are thought to be mediated by bone marrow–derived dendritic cells in the thymus medulla and pharyngeal pouch–derived epithelium in the cortex (16–18).

Studies with nucleoside precursors ([^{3}H]TdR, BUdR) have shown that cell proliferation in the thymus is extensive (19–24). However, >95% of the cells that synthesize DNA do not leave the thymus and presumalby die there (19, 25). The dying cells are double-positive [$CD4^+$, $CD8^+$] cortical thymocytes (21, 25). This extent of thymocyte death is not readily explained by the deletion of self-reactive clones. We have isolated a population of $CD4^+$ $CD8^+$ cells that are killed in culture by phagocytosis into macrophages. The susceptible thymocytes have rearranged TCR-α and -β genes, express high levels of TCR transcripts, but have low levels of surface TCR and CD3. These findings suggest to us that thymocyte turnover in situ reflects killing of cells that fail to form TCR that can be positively selected, presumably by thymic MHC products.

Materials and Methods

Reagents. Reagents used were RPMI 1640 and trypan blue (Gibco Laboratories, Grand Island, NY); FCS (Sterile Systems, Logan, UT); PMA and A23187 ionophore, (Sigma Chem-

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ical Co., St. Louis, MO); Con A, (Sigma Chemical Co., and Pharmacia Fine Chemicals, Piscataway, NJ); Percoll (Pharmacia); collagenase (CLS II; CooperBiomedical, Malvern, PA); murine rIL-1α (Dr. P. LoMedico, Hoffman LaRoche, Nutley, NJ); rIL-4 from Hela cells transfected with the murine IL-4 gene (26); human rIL-1α (Dainippon Pharmaceutical, Osaka, Japan); human rIL-2 (Biogen, Cambridge, MA). Phycoerythrin anti-CD4 (PE clone GK1.5), FITC-anti-CD8 (clone 53-6.7), and FITC-anti-Thy-1 (clone 30H12) were purchased from Becton Dickinson & Co. (Mountain View, CA) or were prepared by Dr. Y. Katsura (Kyoto University); a panel of standard mAbs, described elsewhere (27), were obtained from the American Type Culture Co. (Bethesda, MD) or from colleagues.

Mice. (C57BL/6 × DBA/2)F_1 and (BALB/c × DBA/2)F_1 mice, 5–8 wk old and of both sexes were purchased from the Trudeau Institute, Saranac Lake, NY, or from Shizuoka Agricultural Cooperative Association for Laboratory Animals, Shizuoka, Japan.

Culture Medium. RPMI 1640 was supplemented with 5% FCS, 20 μg/ml gentamicin, and 5×10^{-5} M 2-ME.

Flow Cytometry. Single-color labeling involved a panel of rat anti-mouse leukocyte mAbs (27) followed by RG7 monoclonal mouse anti-rat Ig (28), supplied by Dr. E. Pure (The Rockefeller University) and FITC-goat anti-mouse Ig (Scandic, Vienna, Austria). The hamster mAb 2C11 anti-CD3 (29) was visualized with the same system; S4B6 hamster anti-murine IL-2 (30) was the negative control. 10,000 cells were analyzed per sample. Samples had <5% nonviable cells and were gated on the basis of low forward and right angle light scatter.

Thymocytes and Thymocyte Subpopulations. Thymi were dissociated with fine forceps. The suspension was applied to stainless steel sieves in culture dishes and then disrupted with the rubber end of a syringe plunger. The cells were collected in RPMI 1640 in 15-ml conical polypropylene tubes (Sarstedt Inc., Newton, NC), centrifuged at 4°C, resuspended in 1.5 ml RPMI 1640 per group of one to three thymi (~20×10^7 cells), passed over nylon wool columns, resuspended again as above, and applied to gradients consisting of 3 ml each of 63%, 54%, and 45% vol/vol Percoll. The 63% Percoll consisted of seven parts Isoperc (nine parts Percoll, Pharmacia, one part 10× HBSS), one part FCS, and two parts PBS. The tubes were then spun at 1,000 *g* for 25 min at 4°C in an RC3b Sorvall centrifuge, swinging bucket rotor, using slow acceleration and no brake.

Three fractions were collected: a high density pellet, a medium density 60–70% interface, and a low density 50–60% interface. The cells were washed three times in RPMI 1640 before further analysis. With respect to standard surface markers of murine thymocytes, virtually all of the high density thymocytes had high levels of Thy-1, CD5, CD4, CD8, CD11a antigens and low levels of MHC Class I products and IL-2-R (Fig. 1, *bottom panels*). Weak but clear staining with anti-CD3 was evident, but reactivity with the KJ16 anti-TCR reagent was not detected. The low density thymocytes were Thy-1$^+$, CD5$^+$, CD4$^+$, or CD8$^+$, LFA-1 or CD11a$^+$ (Fig. 1, *top panels*). Most low density cells had high levels of class I and low levels of class II MHC products. 70–80% stained strongly with anti-CD3, i.e., at the same level as peripheral T cells, while 15–20% reacted with an anti-TCR reagent KJ 16 (Fig. 1). The KJ 16 TCR is encoded by the Vβ8 subfamily of TCR Vβ genes and is expressed on some 20% of mature T cells in appropriate mouse strains (31). Medium density thymocytes resembled the high density cells in phenotype (not shown).

Two-color labeling for CD4 and CD8 antigens (PE-GK 1.5 and FITC-53-6.7, respectively) confirmed that high and medium density cells were >95% double-positive. The low density cells were only 20% double-positive, and 30–45% either CD4$^+$ or CD8$^+$ (not shown).

Southern and Northern Blotting. As described previously (32), high molecular weight DNA was extracted, cleaved with Eco RI, electrophoresed on 0.7% agarose gels, and transfered to nitrocellulose. DNA probes were labeled by nick translation with ^{32}P. To detect TCR gene rearrangement we used a $J\alpha_1$ probe from the most 5′ region of the Jα cluster. The probe was a 3.5-kb Eco RI/Hind III fragment of the Jα cosmid clone TA 28.1 (33); it hybridized to an 8.4-kb Eco RI germline fragment. The Jβ1 probe was a 2.0-kb Pst I fragment, containing Jβ1.1–1.5 sequences from a TCR-β cosmid clone 2.3W7 (34, 35); it hybridized to a 10.5-kb Eco RI germline fragment.

Cytoplasmic RNA was extracted with phenol chloroform but adequate samples were only obtained in three of six experiments. Briefly, cells were rinsed with phosphate buffer and

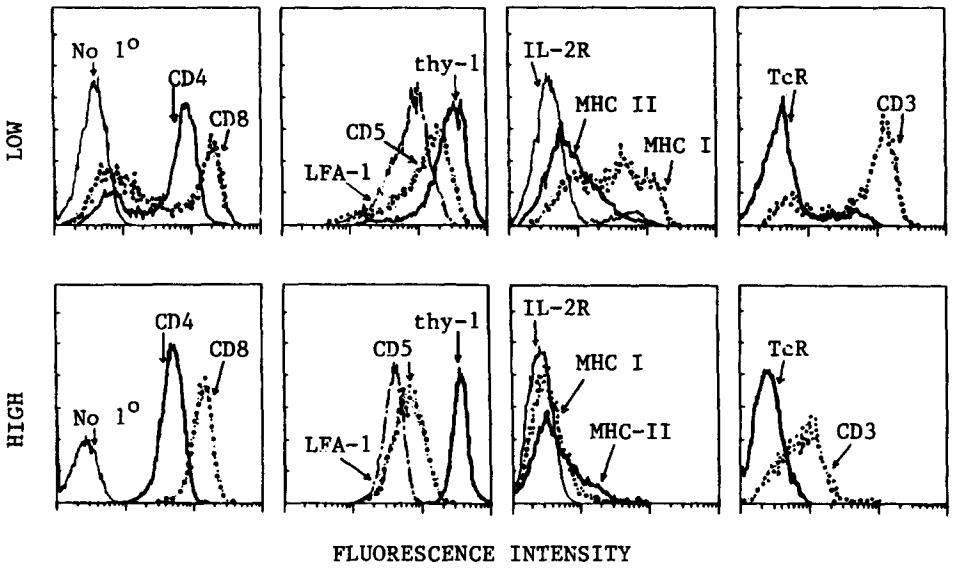

FIGURE 1. Cell surface phenotype of low and high density thymocytes. Percoll gradient fractions were stained with mAbs followed by mouse anti-rat Ig and FITC-goat anti-mouse Ig. The specificities of the primary mAbs (27) are indicated and include: B5-3 anti-Thy-1; 53-7.3 anti-Lyt-1, CD5; GK 1.5 anti-CD4; 53-6.7 anti-Lyt-2, CD8; M1/42 anti-H-2K$^{\text{all haplotypes}}$, MHC class I; M5/114 anti-I-A/E MHC class II; 2C11 anti-CD3; and KJ 16 anti-Vβ8, TCR. Other mAbs that proved nonreactive with both populations (not shown) were 2.4G2 anti-Fc receptor; 33D1 anti-dendritic cell; F4/80 antimacrophage; NL145 anti-interdigitating cell; RA3-3A1, anti-B220; M1/70 anti-C3bi receptor.

suspended in lysis buffer (140 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris, pH 8.5, 10 mM vanadyl ribonucleoside complex) with 0.1% NP-40. The cells were vortexed briefly, incubated on ice 5 min, and centrifuged. 1 vol of proteinase K buffer (0.2 M Tris, pH 7.4, 25 mM EDTA, 0.3 M NaCl, 2% SDS, 0.4 mg/ml proteinase K) was added to the supernatants, and the mixture was incubated for 30 min at 37°C. Samples were extracted with phenol and chloroform-isoamylalcohol (24:1). To the extracts we added three volumes of ethanol to precipitate the RNA. The RNA was treated with glyoxal and DMSO at 50°C for 1 h and electrophoresed in agarose containing 10 mM sodium phosphate buffer, pH 7.0, followed by transfer to nitrocellulose filters. In other experiments, which are the ones shown in the Results, poly(A) RNA (36) was isolated and probed. The yields of poly(A)/total RNA were 3.1/200 μg for 10^9 high density cells; 42/1,400 μg for 5×10^8 medium density cells; and 29.4/1,300 μg for 4×10^8 low density cells. The TCR probes used for hybridization were: Cα, a 540-bp Sau 3A1 fragment of the TT11 cDNA clone (37); Cβ, a 730-bp Eco RI fragment of the 86T5 cDNA clone (38); Vβ8.3, a 300-bp Eco RI-Hap II fragment of the TB23 cDNA clone (39); Dβ, a mixture of a 1.7-kb Pst I fragment from subclone pUC8-Dβ1 and a 2.4-kb Hind III/Eco RI fragment from subclone pUC8-Dβ2 (35); Vα4, a 275-bp Eco RI-Hind III fragment from the TA65 cDNA clone (40).

Macrophages and Dendritic Cells. Peritoneal cells were allowed to adhere for 6–9 h and the nonadherent fraction was removed by gently pipetting. 25–30% of the cells attached as a macrophage-enriched monolayer. Splenic dendritic cells were enriched to >90% purity as described (41). Monolayers of adherent thymic macrophages and dendritic cells were prepared from collagenase-digested thymi (42).

T Cell Mitogenesis. 3×10^5 thymocytes were cultured in microtest wells in triplicate. The stimuli were graded doses of accessory macrophages or dendritic cells, growth factors, and

mitogens that are indicated in the Results. Proliferation was measured by [^{3}H]TdR uptake as in the Results.

Binding and Uptake of Thymocytes to Macrophages. High density or nylon wool nonadherent thymocytes were added to culture vessels containing adherent macrophages from the peritoneal cavity or thymus. About 10^6 thymocytes and 10^5 macrophages were cocultured/100-mm^2 surface area. Cell viability was monitored by counting trypan blue–negative cells in cultures that were harvested with Pasteur pipettes. To examine the binding and uptake of thymocytes to macrophages, thymocytes were allowed to form rosettes by culturing for 1–2 h at 37°C. The coverslips with attached cells were removed and rinsed in a beaker of medium. The coverslips were stained on ice with FITC–anti-Thy-1 which would stain attached but not ingested T cells. Alternatively, the preparations were fixed in 2% EM-grade glutaraldehyde in 0.1 M sodium cacodylate buffer with 0.1 M sucrose overnight and examined by phase contrast, scanning, and transmission electron microscopy (EM) described (43).

Results

Immunologic Function of Thymocytes Separated on Percoll Gradients. Our experiments began as a study of the accessory cell requirements for thymocyte proliferation in culture. Since it was known that the thymus contains a majority of immunologically *unresponsive* cells, we attempted to separate functionally active and inactive subpopulations using Percoll density gradients. We isolated three fractions of thymocytes and monitored responses to a panel of stimuli in the presence of accessory macrophages or dendritic cells. In the first seven experiments, the mean yields of high, medium, and low density cells were 24 ± 3, 59 ± 4, and 17 ± 2% of total thymocytes, respectively.

The low density fraction contained most of the thymocytes that could proliferate to the mitogens we tested (Table I). The stimuli included: anti-CD3 mAb and macrophages, Ca^{2+} ionophore and PMA, interleukins, and dendritic cells with alloantigens or Con A. Medium density cells responded weakly, and high density cells did not respond at all (Table I). The lack of a response by high density thymocytes was not an artifact of the Percoll density gradient because similarly prepared, high density spleen T cells responded to lectin and alloantigens. Also, the results are consistent with prior work showing that double-positive thymocytes, like the medium and high density fractions (Materials and Methods; Fig. 1), respond weakly to many stimuli (44–47).

High Density Thymocytes Are Killed by Macrophages in Culture. When we monitored cell recoveries during the course of the above mitogenesis assays, we noted marked differences depending on the type of accessory cell and fraction of thymocytes. After 16 h of coculture with macrophages, only 11 ± 5.7% of the high density cells (six experiments) were recovered relative to controls without macrophages. In contrast, 88 ± 3% of the low density cells survived after overnight culture with macrophages. Thymocyte viability was not affected by coculture with dendritic cells. A dose of 3×10^6 macrophages was needed to kill most thymocytes in cultures of 20×10^6 high density cells in a day. When bulk thymus cells were cultured with macrophages, viable cell recovery dropped to 37 ± 6%. The loss of viable high density cells was accelerated in the presence of either Con A or PMA, whereas low density cells expanded in numbers when the macrophages were added together with Con A or with anti-CD3 mAb (not shown).

Qualitatively similar results were obtained with thymic macrophages and den-

TABLE I

Responsiveness of Low and High Density Thymocytes to Cytokines and Mitogens

Stimulus	Proliferative response ([^{3}H]TdR uptake) by thymocyte fractions		
	Low density	Medium density	High density
	cpm × 10^{-3}		
None	0.09	0.07	0.07
Human rIL-1α (100 U/ml)	3.4	0.08	0.06
Human rIL-2 (1 U/ml)	142	9.3	0.06
Murine rIL-4 (10 U/ml)	26	1.4	0.11
αCD3, 2C11 mAb	0.08	0.06	0.08
αCD3 + 10^4 peritoneal exudate cells	61	11	0.15
PMA (5 ng/ml)	0.23	0.05	0.06
A23187 (250 ng/ml)	0.09	0.08	0.06
PMA + A23187	149	52	0.20
Syngeneic DC (3 × 10^3)	0.3	0.10	0.10
Allogeneic DC (3 × 10^3)	82	0.40	0.30
Syngeneic (3 × 10^3) + Con A (1 μg/ml)	71	0.20	0.10

Nylon wool nonadherent thymocytes were fractionated into low, medium, and high density populations and cultured with the indicated stimuli at 3 × 10^5 cells/flat micro test well. [^{3}H]TdR uptake was measured at 66–72 h with 4 μCi/ml. Data are means of triplicates where standard deviations are <10%.

dritic cells. The macrophages killed thymocytes, particularly high density thymocytes, while the dendritic cells did not (not shown).

The Selectivity of Macrophage-mediated Killing. Two-color FACS analyses with anti-CD4 and -CD8 mAbs were performed of *bulk* or high density thymocytes that had been cultured with or without macrophages. Bulk thymocytes contain double-negative, single-positive, and double-positive subpopulations, but the macrophages only depleted double-positive, $CD4^+/CD8^+$ cells (Fig. 2, *top*; Table II). The double-positive cells that were cultured in the absence of macrophages formed two populations; only the population with lower amounts of CD4 and CD8 (Fig. 2, *arrow*) were killed by the macrophages. FACS analyses of the high density thymocyte subpopulations showed that they consisted primarily (>80%) of these "low" double-positive cells (not shown).

One-color studies with anti-CD3 were also performed. The macrophages selectively killed CD3-low cells (Fig. 2 *C*). As a result, the cultures of thymocytes were enriched in CD3-hi thymocytes (Fig. 2 *C*). Therefore, macrophages kill double-positive, CD3-low cells primarily.

Phagocytosis as the Mechanism of Thymocyte Killing. Light microscopy revealed that thymocyte killing entailed a phagocytic mechanism. When peritoneal or thymic macrophages were "pulsed" with high density or bulk thymocytes for 1 h and then washed, the macrophages but not dendritic cells were rosetted with T cells. In Fig. 3, this selective rosetting is shown for heterogenous populations of adherent thymic macro-

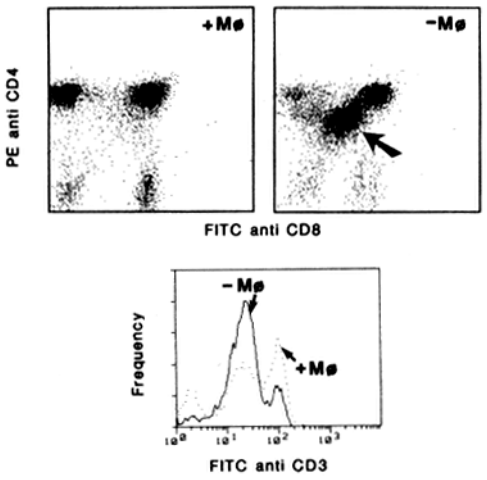

FIGURE 2. Depletion of $CD4^+$, $CD8^+$, $CD3^{low}$ thymocytes after coculture with macrophages. 2 × 10^7 unfractionated thymocytes were cultured in 35-mm wells for 16 h with or without 3 × 10^6 peritoneal macrophages. Viable cell yields (trypan blue-negative) were 50 and 15% of starting levels, respectively. The cells were stained with PE-anti CD4 and FITC-anti CD8 (top) to show that coculture with macrophages [+Mφ] removed a subpopulation of double-positive thymocytes and enriched for single-positive and double-negative thymocytes. The same cells (bottom) were stained with 2C11 hamster anti-mouse anti-CD3 mAb (29) to show that CD3-low cells (many double-positive cells have low levels of CD3; e.g., see Fig. 1) were selectively removed by macrophages.

phages and dendritic cells (Fig. 3 *a*), and for enriched populations of thymic macrophages (Fig. 3 *b*).

If FITC-anti-Thy-1 was added at 4°C to macrophage-thymocyte rosettes, all the lymphocytes were labeled (Fig. 3 *d*). Over a period of 3-4 h at 37°C, most of the T cells in the rosettes were internalized into phagocytic vacuoles (Fig. 3, *e* and *f*). The ingested lymphocytes were not accessible to FITC-Thy-1 (Fig. 3 *f*), and were degraded in a matter of hours (not shown).

Electron microscopy provided a high resolution view of the binding and internalization process. By scanning EM, as many as 10 or more thymocytes could bind to the surface of each phagocyte (Figs. 4, *A* and *B*). The attached thymocytes appeared viable during the entire process of phagocytosis. This involved the rising up of macrophage membrane over the thymocyte (Figs. 4, *C* and *D*), forming cup-like structures and eventually capturing the T cell beneath protuberances of the macrophage

TABLE II

Macrophages Selectively Deplete Double-Positive Thymocytes

Fraction of thymocytes	Number of viable cells (× 10^{-6}) in cultures (percent of viable cells)			
	Exp. 1		Exp. 2	
	Without MØ	With MØ	Without MØ	With MØ
$CD4^+$ $CD8^-$	1.2 (9)	1.2 (21)	1.7 (19)	1.4 (37)
$CD4^-$ $CD8^+$	0.27 (3)	0.31 (7)	0.52 (6)	0.48 (13)
$CD4^-$ $CD8^-$	0.18 (2)	0.24 (5)	0.26 (3)	0.26 (7)
$CD4^+$ $CD8^+$	7.2 (86)	2.3 (67)	6.3 (72)	1.7 (44)

2 × 10^7 nylon wool nonadherent thymocytes were cultured in 35-mm dishes with or without 3 × 10^6 resident peritoneal macrophages for 16 h in 5% FCS, RPMI 1640, and 50 μM 2-ME. The nonadherent cells were harvested, a viable count was taken, and the cells stained for CD4 and CD8 antigens as in Fig. 5. The two-color FACS plots were analyzed for the percentage of cells in each of the major subpopulations of thymocytes, and these data are in parentheses. The viable cell yield is total trypan blue-negative cells in the culture × percentage of viable cells in each fraction.

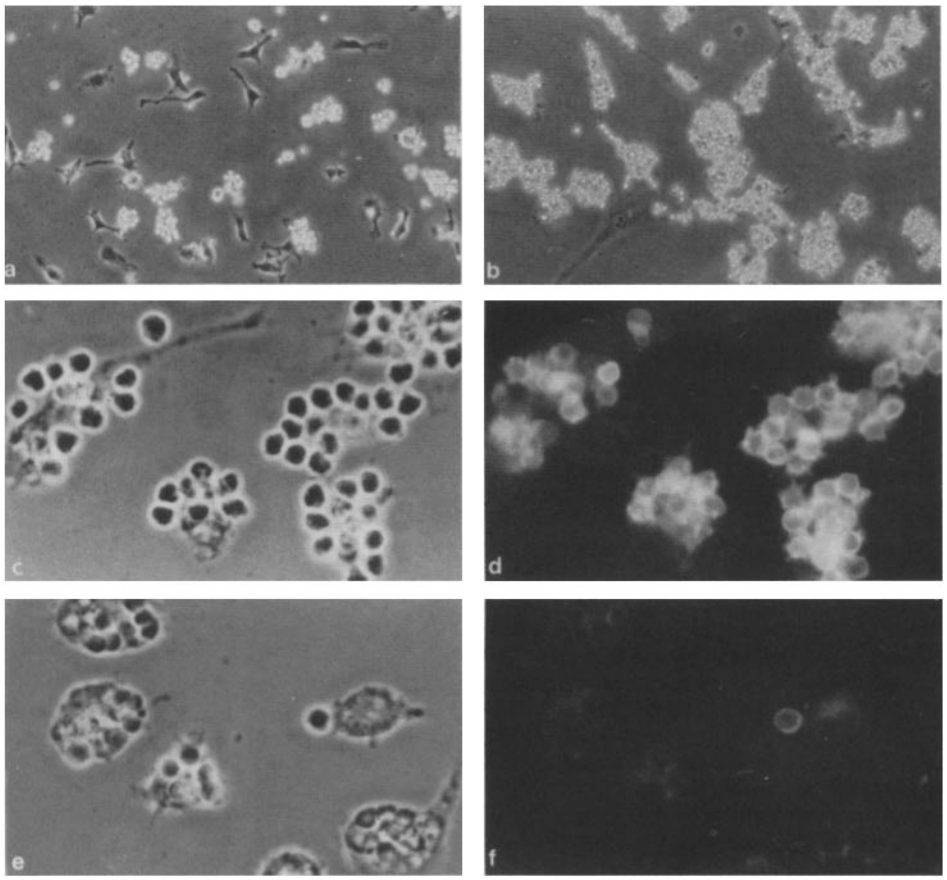

FIGURE 3. Binding and internalization of high density thymocytes. 3×10^7 thymocytes were added to 60-mm plastic dishes containing 12-mm circular coverslips to which $1-3 \times 10^4$ adherent cells had been attached. After 1 h at 37°C, the coverslips were rinsed of nonattached thymocytes by dipping each in a small beaker of medium. The macrophages were "rosetted" with thymocytes (*a–d*). The coverslips were returned to culture at 37°C, whereupon phagocytosis of most thymocytes occurred in 3–6 h (*e*, *f*). (*a*) Rosetting of thymocytes about thymic, low density, adherent cells released by collagenase digestion (42). Rosettes form about macrophages but not dendritic cells. ×240. (*b*) Same as *a*, but the low density thymus adherent cells were cultured for 1 d before testing. During this culture, the dendritic cells eluted from the adherent monolayer, leaving behind macrophages and a rare fibroblast. ×240. (*c*) Higher power of thymocytes rosetted about peritoneal macrophages. ×950. (*d*) Same as *c* but stained with FITC anti-Thy-1 mAb for 1/2 h at 4°C. (*e*) Phagocytosis of rosetted thymocytes after culture at 37°C for 3 h to produce "tingible body" macrophages. The term "tingible bodies" refers to the inclusions, which are phagosomes of lymphocytes (Fig. 5). ×950. (*f*) Same as *e*, but stained with FITC anti-Thy-1, applied as in *d*. The phagocytosed lymphocytes were not accessible to anti-Thy-1. The macrophage cytoplasm had a low level of background cytoplasmic fluorescence, which outlined the thymocyte-laden phagosomes.

surface (Figs. 4, *C* and *E*). The phagocytosed cells were digested rapidly since most of the protuberances had disappeared within 5–6 h of uptake (Fig. 4 *F*).

By transmission EM, intact thymocytes initially attached to the cell surface (Fig. 5 *A*). The thymocytes appeared intact during and after uptake into phagocytic vacuoles

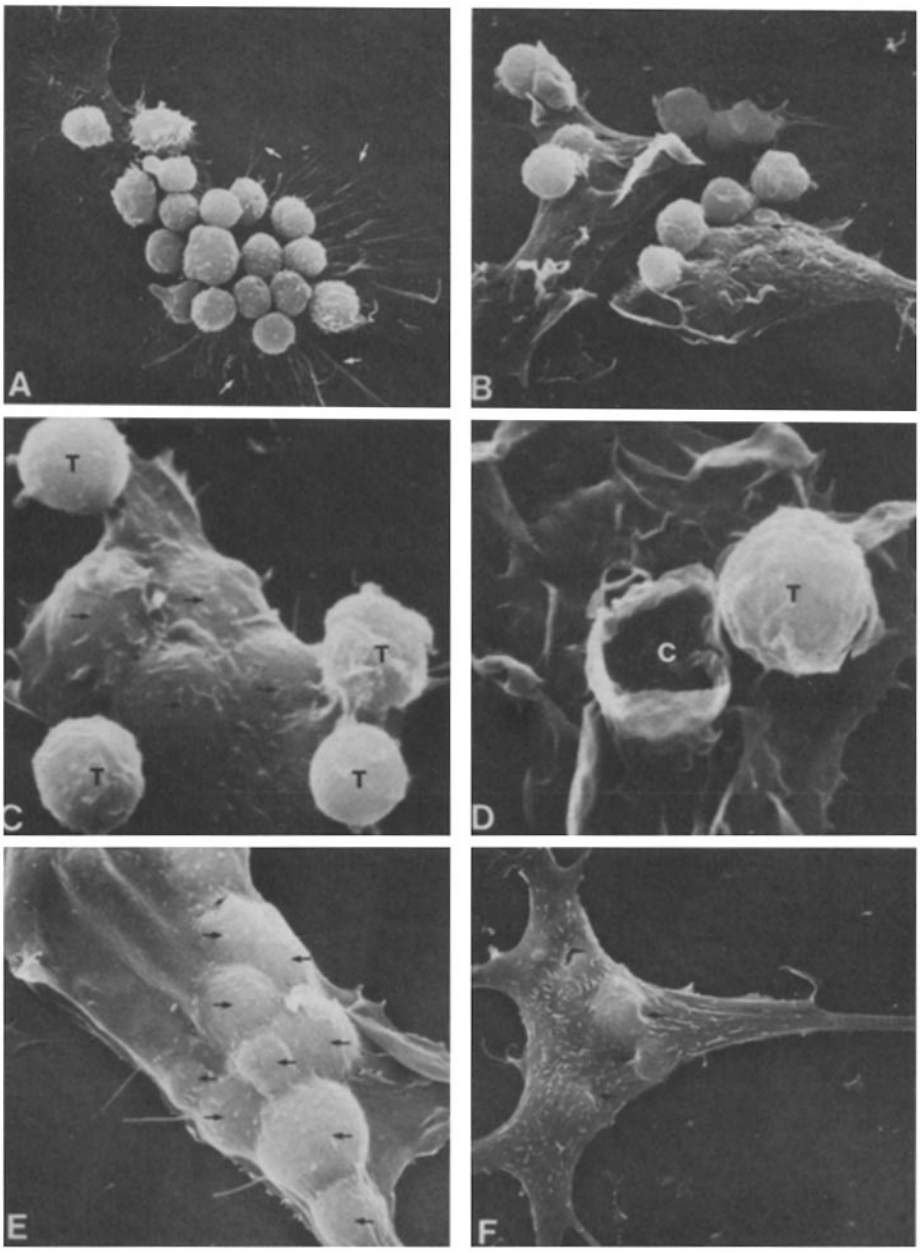

FIGURE 4. Scanning EM of thymocytes rosetted to, and ingested by, peritoneal macrophages. Thymocytes were added to macrophages on coverslips, the nonattached cells were washed away, and the thymocyte-macrophage rosettes were either fixed or returned to culture at 37°C before fixation, as indicated. (*A*) Attachment for 3 h at 4°C. Small round thymocytes are bound to much of the macrophage surface. The presence of long thin macrophage processes reflects the culture at low temperature. ×2,100. (*B*) Attachment for 3 h at 37°C. The ingestion of some of the thymocytes results in the formation of protuberances (*arrows*) of the macrophage surface. Other thymocytes are attached to the surface. ×2,500. (*C*) Same as *B*, with four ingested [arrows] and four attached thymocytes (T). ×5,000. (*D*) Attachment for 90 min at 37°C followed by culture for 1 h. A select view of two presumptive, forming phagocytic vacuoles. A thymocyte (T) is present in one forming phagosome (*arrow*), while the T cell in the other (*C*) has presumably been washed away during processing. ×7,400. (*E*) Attachment for 90 min at 37°C followed by culture for 2 h. The outlines of at least nine ingested thymocytes is evident (*arrows*). ×9,500. (*F*) Attachment for 90 min at 37°C followed by culture for 4 h. Most of the ingested thymocytes have been digested so that the bulges on the macrophage surface have been reduced (*arrows*), and the phagocytes are all well spread. ×2,500.

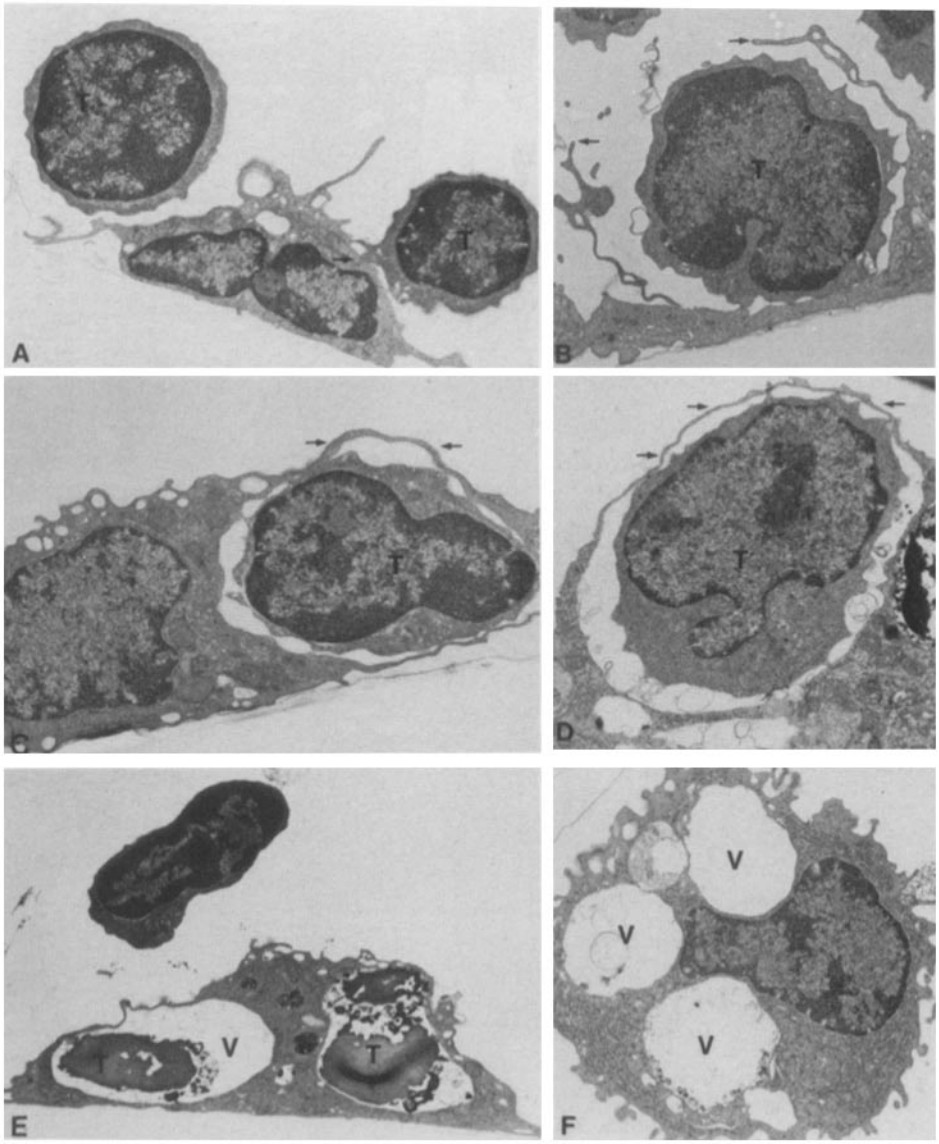

FIGURE 5. Transmission EM of thymocyte recognition and uptake by macrophages. Specimens comparable to those in Fig. 2 were sectioned and examined by transmission EM. (*A*) Attachment (*arrow*) of thymocytes (*T*) to the macrophage. ×6,600. (*B*) Onset of the phagocytic event. Macrophage pseudopods (*arrows*) begin to surround the thymocyte. ×8,300. (*C* and *D*) Completion of the phagocytic event. Thymocytes, apparently viable, are enclosed within phagocytic vacuoles resulting in a "bulging" of the cell surface (*arrows*) as noted by scanning EM (Fig. 4). (*C*) ×6,000. (*D*) ×8,300. (*E*) Degradation of internalized thymocytes to form "tingible bodies" (*V, T*). ×4,000. (*F*) Extensive digestion of the thymocytes leaves lucent vacuoles (*V*), which eventually shrink (not shown), leaving no remnant of the phagocytic meal. ×6,000.

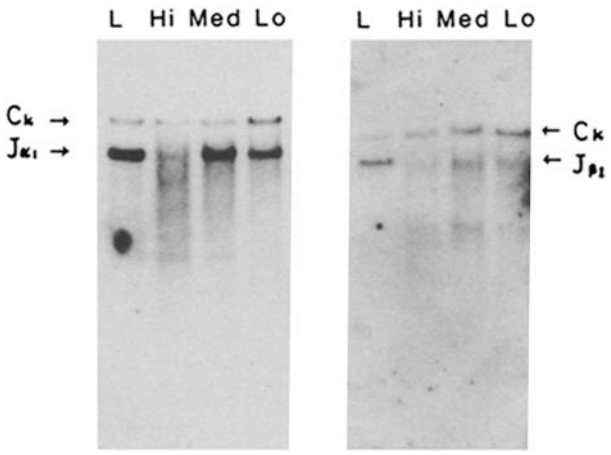

FIGURE 6. Southern blotting to detect rearrangement of TCR-α and -β genes in thymocytes fractionated on dense Percoll gradients. DNA was extracted from liver (*L*), and from thymocytes that had been separated into high (*Hi*), medium (*Med*), and low (*Lo*) density cells. Eco RI-cleaved DNA was probed with a C_κ Ig gene probe (60) as an internal standard, since the 17-kb germline Ck fragment should not rearrange in T cells. To detect rearrangement, 5′ $J\alpha_1$ and $J\beta_1$ TCR probes were used. Note that germline 8.4-kb α and 10.5-kb β fragments were not detected in the high density thymocytes, which were rearranged to yield Eco RI fragments of smaller size.

(Fig. 5, *B–D*). Extensive condensation and digestion then occurred, forming "tingible bodies" (Fig. 5 *E*). Eventually, electron lucent vesicles were noted (Fig. 5 *F*), followed by vacuole shrinkage and resumption of the control macrophage morphology (not shown).

Using an earlier approach for determining if thymocyte killing would occur extracellularly *before* phagocytosis (48), we added trypan blue to living cultures. None of the ingested thymocytes were trypan blue–positive. This indicated that the killing of thymocytes followed rather than preceded phagocytosis. As a control, we applied heat-killed thymocytes (56°C, 1/2 h) that were opsonized with anti-Thy-1 mAb. In the presence of trypan blue, most of the phagocytosed cells were trypan blue–positive because cell death had preceded uptake (not shown).

Southern and Northern Analyses of TCR DNA and RNA. When we analyzed the rearrangement and expression of TCR-α and -β genes, the high density macrophage-sensitive thymocytes gave an unusual result. We used Jα and Jβ probes that detect the most 5′ segments of the TCR genes (see Materials and Methods). Southern blots with these probes showed a near absence of germline DNA in the high density fraction (Fig. 6), indicating that the vast majority of cells had undergone a rearrangement that would delete or alter the size of the Eco RI Jα and Jβ restriction fragments on both chromosomes. Both germline and rearranged TCR genes were noted in the medium and low density fractions (Fig. 6).

A possible explanation for the lack of germline DNA in the high density cells would be that the thymocytes had not productively rearranged TCR genes. However, Northern analysis of total cytoplasmic RNA or poly(A) selected RNA with TCR region probes identified a 1.7-kb α message, and 1.3-kb β transcripts (Fig. 7), which are the appropriate sizes for full-length TCR-α and -β gene products (9, 35). Probes for Vα (not shown) and Vβ (Fig. 7) segments also hybridized with these transcripts, validating their full-length nature. Thus high density cells are in effect fully "mature" with respect to TCR gene rearrangement and transcription but fail to form normal levels of surface TCR and CD3.

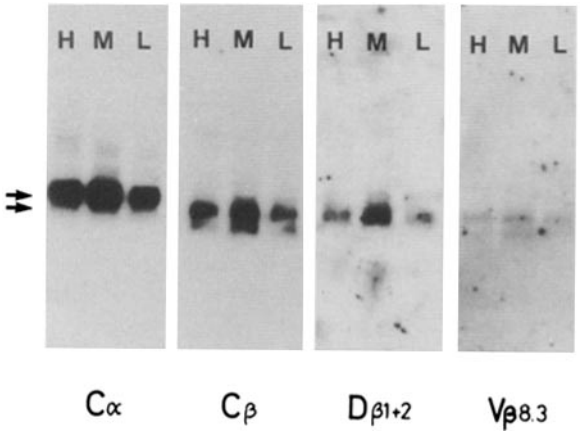

FIGURE 7. DNA/RNA hybridization for TCR mRNA in thymocytes that had been fractionated on dense Percoll gradients. In the two left panels, poly(A)RNA was hybridized with Cα and Cβ probes (12-h exposures), while in the right panels, RNA was hybridized with a mixture of Dβ1 and β2 probes or with Vβ8.3 (3- and 7-d exposures).

Discussion

Phagocytosis of Double-Positive Thymocytes. A subpopulation of $CD4^+$ $CD8^+$ thymocytes is bound and phagocytosed by mouse macrophages in culture. This phenomenon might be related to the extensive known turnover of newly formed (labeled with DNA precursors) thymocytes in situ (see Introduction), for which a mechanism has not been apparent previously. We have not been able to identify a ligand or mAb that reproducibly blocks the binding and uptake of thymocytes in culture.

It is not clear if the macrophage is simply scavenging thymocytes that are already damaged in some way, or whether the thymocyte is viable but carries ligands that induce receptor-mediated phagocytosis. We favor the latter for the following reasons. (*a*) There is no evidence for a gross toxic factor in our isolation procedure, since we find no binding or phagocytosis of other subpopulations of thymocytes (Fig. 2, Table II), nor peripheral T cells handled similarly (not shown). (*b*) The thymocytes that were phagocytosed did not label with trypan blue that was added continuously to the culture. This should label dying cells if death preceded phagocytosis (48). (*c*) The fine structure of the ingested cells (Figs. 4 and 5) seemed intact by scanning and transmission EM. (*d*) The ingested thymocytes exhibited unusual features in the expression of TCR-α and -β genes, which suggests that these cells had a circumscribed abnormality in this critical receptor for T cell function. (*e*) There is evidence that viable B lymphocytes are phagocytosed by macrophages in situ, perhaps by a similar process. These latter two points will be discussed in more detail.

TCR Gene Rearrangement and Expression in Double-Positive Thymocytes. The availability of a high density, T cell fraction that was phagocytosed efficiently by macrophages revealed interesting features of the CD3-TCR complex in macrophage-sensitive thymocytes. Rearrangements of both TCR-α and -β alleles had occurred, since the most 5′ Jα and Jβ segments were not in germline configuration (Fig. 6). Such rearrangements are late markers of T cell differentiation (8–12). Double-positive cells also contained high levels of TCR-α and -β full-length transcripts, some of which could be hybridized with Vα and Vβ probes (Fig. 7).

In spite of what appeared to be productive gene rearrangement, surface CD3 was

found in very low amounts, and cell surface TCR were not detectable (Fig. 1). Cell surface CD3 and TCR expression is regulated coordinately (49), so that small amounts of TCR-β protein may have been present on these cells. Farr et al. (50) have studied cortical thymocytes in situ, which are primarily double-positive cells, and shown that an anti-TCR Vβ mAb can label the cell surface and the perinuclear cistern. Roehm et al. (51) and Hannum et al. (51) found that the rate of synthesis and turnover of TCR-β protein is high in double-positive thymocytes, but very little is detected on the cell surface (51, 52). Together, the data suggest that the thymocytes that are killed by macrophages have TCR-α/β transcripts and may be synthesizing TCR-β protein, but for some reason, very little TCR is expressed on the cell surface.

Why might so many double-positive cells have low levels of surface CD3 and TCR in spite of a capacity to rearrange TCR genes on both chromosomes and to produce high levels of transcript? We propose that high expression of a clonotypic TCR on the cell surface requires some interaction of a few TCR molecules on the developing thymocyte with MHC products on accessory cells, presumably the epithelial cells that populate the cortex. If the TCR fail to interact with cortical MHC products, or MHC-peptide complexes (53), surface expression of the intrathymocyte transcripts may not be selected, and the T cell may attempt another rearrangement. The antigen receptors of mature thymic-processed lymphocytes have a remarkable bias for MHC products, especially self-MHC in association with "nominal" antigens (14, 15). In contrast, very few B lymphocytes recognize MHC-antigen complexes (54). If most TCR gene rearrangements encode TCR that either do not recognize self MHC or self MHC-peptide complexes, some selective process must account for the observed skewing of the final T cell repertoire. Therefore, we postulate that developing thymocytes need to produce a receptor with some affinity for self MHC-peptide complexes to stop rearrangement of TCR genes and eventually to avoid killing by macrophages. The low levels of CD3 noted on susceptible thymocytes (Figs. 1 and 2) may be required to signal the thymocyte that a productive thymocyte–accessory cell interaction has occurred.

Analogies of Double-Positive Thymocytes and Germinal Center B Cells. Macrophage-thymocyte cocultures contain many profiles termed "tingible body macrophages" (Figs. 3 and 5). Tingible bodies are DNA^+ inclusions due to ingested cells. One locale where such macrophages normally are prominent in situ is the germinal center of peripheral lymphoid organs (55). The tingible bodies are B lymphocytes that have incorporated DNA precursors just *before* (30 min or more) phagocytosis (56). Therefore, germinal center B cells, like many double-positive thymocytes, seem to be viable cells that are eliminated shortly after synthesizing DNA. Another similarity is that cortical thymocytes and germinal center B cells both react strongly with the galactose-specific peanut agglutinin (57).

It has been reasoned that germinal centers are the site in which there is extensive somatic mutation in proliferating B cells to produce memory B cells with Igs of higher affinity (58). Somatic mutation may yield high affinity clones, but it is striking that in primed mice there are relatively few cells that fail to bind antigen (59). If somatic mutation is occurring in the germinal center, there may be mechanisms for selecting antigen-binding mutants and eliminating nonbinding ones. Immune complexes, retained extracellularly on the surface of follicular dendritic cells (55), might provide the pressure to select antigen-reactive clones, while tingible body macrophages would

provide a clearance mechanism for mutants that are not antigen reactive. We propose that macrophages in germinal centers and in the thymus clear viable lymphocytes that are not positively selected by immune complexes or self MHC products, respectively.

Summary

The thymus gland is important for the formation of competent T lymphocytes. However, there is long-standing evidence that >95% of newly formed thymocytes do not emigrate to peripheral lymphoid tissues but instead die locally. We have identified a rapid and selective pathway for thymocyte turnover in vitro. The mechanism entails binding, uptake, and digestion by macrophages. The susceptible cells are a subpopulation of double-positive thymocytes. These thymocytes can be enriched by virtue of their high buoyant density in Percoll and prove to have low levels of surface CD3 and little or no surface TCR. However TCR-α and -β genes have undergone rearrangement, and full length α and β transcripts are abundant. Therefore many double-positive cells rearrange and express TCR genes but do not have normal levels of TCR on the cell surface. We propose that thymocytes that undergo high turnover in situ are unable to form receptors that can be selected by MHC molecules in the thymus, and that these cells are recognized and cleared by the macrophage.

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References

1. Miller, J. F. A. P. 1962. Effect of neonatal thymectomy on the immunological responsiveness of the mouse. *Proc. R. Soc. Lond. B Biol. Sci.* 156:415.
2. Good, R. A., A. P. Dalmasso, C. Martinez, O. K. Archer, J. C. Pierce, and B. W. Papermaster. 1962. The role of the thymus in development of immunologic capacity in rabbits and mice. *J. Exp. Med.* 116:773.
3. Jankovic, B. D., B. H. Waksman, and B. G. Arnason. 1962. Role of the thymus in immune reactions in rats. I. The immunologic response to bovine serum albumin (antibody formation, Arthus reactivity, and delayed hypersensitivity) in rats thymectomized or splenectomized at various times after birth. *J. Exp. Med.* 116:159.
4. Arnason, B. G., B. D. Jankovic, B. H. Waksman, and C. Wennersten. 1962. Role of the thymus in immune reactions in rats. II. Suppressive effect of thymectomy at birth on reactions of delayed (cellular) hypersensitvity and the circulating small lymphocyte. *J. Exp. Med.* 116:177.
5. Rygaard, J. 1973. *In* Thymus and Self. John Wiley & Sons, New York.
6. Waksman, B. H., B. G. Arnason, and B. D. Jankovic. 1962. Role of the thymus in immune reactions in rats. III. Changes in the lymphoid organs of thymectomized rats. *J. Exp. Med.* 116:187.
7. Parrott, D. M. V., M. A. B. De Sousa, and J. East. 1966. Thymus-dependent areas in the lymphoid organs of neonatally thymectomized mice. *J. Exp. Med.* 123:191.
8. Haars, R., M. Kronenberg, W. M. Gallatin, I. L. Weissman, F. L. Owen, and L. Hood. 1986. Rearrangement and expression of T-cell antigen receptor and gamma genes during development. *J. Exp. Med.* 164:1.
9. Snodgrass, H. R., Z. Dembic, M. Steinmetz, and H. von Boehmer. 1985. Expression of T-cell antigen receptor genes during fetal development in the thymus. *Nature (Lond.).* 315:232.

10. Raulet, D. H., R. D. Garman, H. Saito, and S. Tonegawa. 1985. Developmental regulation of T-cell receptor gene expression. *Nature (Lond.).* 314:103.
11. Born, W., G. Rathbun, P. Tucker, P. Marrack, and J. Kappler. 1986. Synchronized rearrangement of T-cell α and β chain genes in fetal thymocyte development. *Science (Wash. DC).* 234:479.
12. Furley, A. J., S. Mizutani, K. Weilbaecher, H. S. Dhaliwal, A. M. Ford, L. C. Chan, H. V. Molgaard, B. Toyonaga, T. Mak, P. van den Elsen, D. Gold, C. Terhorst, and M. F. Greaves. 1986. Developmentally regulated rearrangement and expression of genes encoding the T cell receptor-T3 complex. *Cell.* 46:75.
13. Kappler, J. W., N. Roehm, and P. Marrack. 1987. T cell tolerance by clonal elimination in the thymus. *Cell.* 49:273.
14. Kast, W. M., L. P. De Waal, and C. J. M. Melief. 1984. Thymus dictates major histocompatibility complex (MHC) specificity and immune response gene phenotype of class II MHC-restricted T cells but not of class I MHC-restricted T cells. *J. Exp. Med.* 160:1752.
15. Fink, P. J., and M. J. Bevan. 1978. H-2 antigens of the thymus determine lymphocyte specificity. *J. Exp. Med.* 148:766.
16. Lo, D., and J. Sprent. 1986. Identity of cells that imprint H-2 restricted T-cell specificity in the thymus. *Nature (Lond.).* 319:672.
17. Ready, A. R., E. J. Jenkinson, R. Kingston, and J. J. T. Owen. 1984. Successful transplantation across histocompatibility barrier of deoxyguanosine-treated embryonic thymus expressing class II antigens. *Nature (Lond.).* 310:231.
18. von Boehmer, H., and K. Schubiger. 1984. Thymocytes appear to ignore class I major histocompatibility complex antigens expressed on the thymus epithelial cells. *Eur. J. Immunol.* 14:1048.
19. Matsuyama, M., M. N. Wiadrowski, and D. Metcalf. 1966. Autoradiographic analysis of lymphopoiesis and lymphocyte migration in mice bearing multiple thymus grafts. *J. Exp. Med.* 123:559.
20. Bryant, B. J. 1972. Renewal and fate in the mammalian thymus: mechanisms and inferences of thymocytokinetics. *Eur. J. Immunol.* 2:38.
21. Penit, C. 1986. In vivo thymocyte maturation. BUdR labeling of cycling thymocytes and phenotypic analysis of their progeny support the single lineage model. *J. Immunol.* 137:2115.
22. Shortman, K., and H. Jackson. 1974. The differentiation of T lymphocytes. I. Proliferation kinetics and interrelationships of subpopulations of mouse thymus cells. *Cell. Immunol.* 12:230.
23. Borum, K. 1973. Cell kinetics in mouse thymus studied by simultaneous use of ^{3}H-thymidine and colchicine. *Cell Tissue Kinet.* 6:545.
24. Claesson, M. H., and N. R. Hartmann. 1976. Cytodynamics in the thymus of young adult mice: a quantitative study on the loss of thymic blast cells and non proliferative small thymocytes. *Cell Tissue Kinet.* 9:273.
25. McPhee, D., J. Pye, and K. Shortman. 1979. The differentiation of T lymphocytes. V. Evidence for intrathymic death of most thymocytes. *Thymus.* 1:151.
26. Noma, Y., P. Sideras, T. Naito, S. Bergstedt-Lindquist, C. Azuma, E. Severinson, T. Tanabe, T. Kinashi, F. Matsuda, Y. Yaoita, and T. Honjo. 1986. Cloning of cDNA encoding the murine IgG1 induction factor by a novel strategy using SP6 promoter. *Nature (Lond.).* 319:640.
27. Witmer-Pack, M. D., J. Valinsky, W. Olivier, and R. M. Steinman. 1988. Quantitation of surface antigens on cultured murine epidermal Langerhans cells: rapid and selective increase in the level of surface MHC products. *J. Invest. Dermatol.* 90:387.
28. Springer, T. A., A. Bhattacharya, J. T. Cardoza, and F. Sanchez-Madrid. 1982. Monoclonal antibodies specific for rat IgG1, IgG2a, and IgG2b subclasss and kappa chain mono-

typic and allotypic determinants. Reagents for use with rat monoclonal antibodies. *Hybridoma.* 1:257.

29. Leo, O., M. Foo, D. H. Sachs, L. E. Samelson, and J. A. Bluestone. 1987. Identification of a monoclonal antibody specific for a murine T3 polypeptide. *Proc. Natl. Acad. Sci. USA.* 84:1374.
30. Mosmann, T. R., H. Cherwinsky, M. W. Bond, M. A. Griedlin, and R. L. Coffman. 1986. Two types of murine helper T cell clones I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* 136:2348.
31. Haskins, K., C. Hannum, J. White, N. Roehm, R. Kubo, J. Kappler, and P. Marrack. 1984. The antigen-specific, major histocompatibility complex-restricted receptor on T cells. VI. An antibody to a receptor allotype. *J. Exp. Med.* 160:452.
32. Ikuta, K., M. Hattori, K. Wake, S. Kano, T. Honjo, J. Yodoi, and N. Minato. 1986. Expression and rearrangement of the α, β, and γ chain genes of the T cell receptor in cloned murine large granular lymphocyte lines. *J. Exp. Med.* 164:428.
33. Winoto, A., S. Mjolsness, and L. Hood. 1985. Genomic organization of the genes encoding mouse T-cell receptor α-chain. *Nature (Lond.).* 316:832.
34. Malissen, M., K. Minard, S. Mjolsness, M. Kronenberg, J. Goverman, T. Hunkapiller, M. B. Prystowsky, Y. Yoshikai, F. Fitch, T. W. Mak, and L. Hood. 1984. Mouse T cell antigen receptor: Structure and organization of constant and joining gene segments encoding the β-polypeptide. *Cell.* 37:1101.
35. Siu, G., M. Kronenberg, E. Strauss, R. Haars, T. W. Mak, and L. Hood. 1984. The structure, rearrangement and expression of D β gene segments of the murine T-cell antigen receptor. *Nature (Lond.).* 311:344.
36. Aviv, H., and P. Leder. 1988. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid cellulose. *Proc. Natl. Acad. Sci. USA.* 69:1408.
37. Chien, Y., D. M. Becker, T. Lindsten, M. Okamura, D. I. Cohen, and M. M. Davis. 1984. A third type of murine T-cell receptor gene. *Nature (Lond.).* 312:31.
38. Hedrick, S. M., E. A. Nielson, J. Kavaler, D. I. Cohen, and M. M. Davis. 1984. Sequence relationships between putative T-cell receptor polypeptides and immunoglobulins. *Nature (Lond.).* 308:153.
39. Barth, R. K., B. S. Kim, N. C. Lan, T. Hunkapiller, N. Sobieck, A. Winoto, H. Gershenfeld, C. Okada, D. Hansburg, I. L. Weissman, and L. Hood. 1988. The murine T-cell receptor uses a limited repertoire of expressed Vβ gene segments. *Nature (Lond.).* 316:517.
40. Arden, B., J. L. Klotz, G. Siu, and L. E. Hood. 1985. Diversity and structure of genes of the α family of mouse T-cell antigen receptor. *Nature (Lond.).* 316:783.
41. Nussenzweig, M. C., and R. M. Steinman. 1980. Contributions of dendritic cells to stimulation of the murine syngeneic mixed leukocyte reaction. *J. Exp. Med.* 151:1196.
42. Crowley, M., K. Inaba, M. Witmer-Pack, and R. M. Steinman. 1989. The cell surface of mouse dendritic cells: FACS analyses of dendritic cells from different tissues including thymus. *Cell. Immunol.* In press.
43. Van Voorhis, W. C., L. S. Hair, R. M. Steinman, and G. Kaplan. 1982. Human dendritic cells: Enrichment and characterization from peripheral blood. *J. Exp. Med.* 155:1172.
44. Ceredig, R., A. L. Glasebrook, and H. R. MacDonald. 1982. Phenotypic and functional properties of murine thymocytes. I. Precursors of cytolytic T lymphocytes and interleukin 2-producing cells are all contained within a subpopulation of "mature" thymocytes as analyzed by monoclonal antibodies and flow microfluorometry. *J. Exp. Med.* 155:358.
45. Scollay, R., S. Jacobs, L. Jerabek, E. Butcher, and I. Weissman. 1980. T cell maturation: thymocyte and thymus migrant subpopulations defined with monoclonal antibodies to

MHC region antigens. *J. Immunol.* 124:2845.
46. Chen, W. -F., R. Scollay, I. Clark-Lewis, and K. Shortman. 1985. The size of functional T-lymphocyte pools within thymic medullary and cortical cell subsets. *Thymus.* 5:179.
47. Paterson, D. J., J. R. Green, W. A. Jefferies, M. Puklavec, and A. F. Williams. 1987. The MRC OX-44 antigen marks a functionally relevant subset among rat thymocytes. *J. Exp. Med.* 165:1.
48. Bennett, B., L. J. Old, and E. A Boyse. 1963. Opsonization of cells by isoantibody in vitro. *Nature (Lond.).* 198:10.
49. Weiss, A., and J. D. Stobo. 1984. Requirement for the coexpression of T3 and the T cell antigen receptor on a malignant human T cell line. *J. Exp. Med.* 160:1284.
50. Farr, A. G., S. K. Anderson, P. Marrack, and J. Kappler. 1985. Expression of antigen-specific, major histocompatibility complex-restricted receptors by cortical and medullary thymocytes in situ. *Cell.* 43:543.
51. Roehm, N., L. Herron, J. Cambier, D. DiGuisto, K. Haskins, J. Kappler, and P. Marrack. 1984. The major histocompatibility complex-restricted antigen receptor on T cells: distribution on thymus and peripheral T cells. *Cell.* 38:577.
52. Hannum, C., P. Marrack, R. Kubo, and J. Kappler. 1987. Thymocytes with the predicted properties of pre-T cells. *J. Exp. Med.* 166:874.
53. Bjorkman, P. J., M. A. Saper, B. Samraoui, W. S. Bennett, J. L. Strominger, and D. C. Wiley. 1987. The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens. *Nature (Lond.).* 329:512.
54. Pestalozzi, B., L. Stitz, and R. M. Zinkernagel. 1987. Monoclonal antibodies against viral determinants are not restricted to the K/D end of the major histocompatibility complex. *J. Exp. Med.* 166:295.
55. Chen, L. L., A. M. Frank, J. C. Adams, and R. M. Steinman. 1978. Distribution of horseradish peroxidase (HRP)-anti HRP immune complexes in mouse spleen, with special reference to follicular dendritic cells. *J. Cell Biol.* 79:184.
56. Fleidner, T. M., M. Kesse, E. P. Cronkite, and J. S. Robertson. 1964. Cell proliferation in germinal centers of the rat spleen. *Ann. NY Acad. Sci.* 113:578.
57. Hood, L. E., I. L. Weissman, W. B. Wood, and J. H. Wilson. 1984. *In* Immunology. Benjamin/Cummings, Reading, MA. 267 and 286.
58. MacLennan, I. C. M., and D. Gray. 1986. Antigen-driven selection of virgin and memory B cells. *Immunol. Rev.* 91:61.
59. Wysocki, L., T. Manser, and M. L. Gefter. 1986. Somatic evolution of variable region structures during an immune response. *Proc. Natl. Acad. Sci. USA.* 83:1847.
60. Max, E. E., J. V. Maizel, and P. Leder. 1981. The nucleotide sequence of a 5.5 kilobase DNA segment containing the mouse κ immunoglobulin J and C region genes. *J. Biol. Chem.* 256:5116.