

A NOVEL ACTIVATION PATHWAY FOR
MATURE THYMOCYTES

Costimulation of CD2 (T,p50) and CD28 (T,p44) Induces Autocrine
Interleukin 2/Interleukin 2 Receptor-mediated Cell Proliferation

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Bone marrow-derived T progenitor cells undergo proliferation and maturation under the influence of the thymic microenvironment (1). Only a small fraction of thymocytes are selected to account for self tolerance, as well as self restriction, and become immunocompetent T cells (2). The molecular and cellular mechanisms for the growth and maturation of thymocytes are poorly understood.

Cell surface phenotype analyses have shown that thymocytes are composed of highly heterogeneous populations. Based on cell surface expression of the TCR accessory molecules CD4 and CD8, thymocytes can be divided into three major subpopulations, which generally are thought to relate to maturation stages. They are the CD2⁺CD1⁻CD4⁻8⁻ (double-negative) cells, CD2⁺CD1⁺CD4⁺8⁺ (double-positive) cells, and CD2⁺CD1⁻CD4⁺8⁻ or CD2⁺CD1⁻CD4⁻8⁺ (single-positive) mature thymocytes. Both double-negative and double-positive thymocytes are immature cells located in the cortical compartment of human thymus (3). Mature thymocytes do not express the cortical specific marker CD1 but express TCR A and B chain subunits associated with the CD3 complex and reside predominantly in the medullary compartment (4). The three major populations can be further subdivided using other cell surface antigens as markers.

All populations of thymocytes can express IL-2-Rs (5), and double-negative as well as single-positive thymocytes are able to secrete IL-2. IL-2 induction in thymocytes requires stimulation by combinations of T cell mitogens: PHA or Con A, PMA, ionomycin, and anti-CD2 antibodies (6). Previous studies have shown that certain combinations of anti-CD2 mAbs can trigger peripheral T cell proliferation in the absence of macrophages (7, 8). In contrast to peripheral T cells, thymocytes are unable to proliferate in response to CD2 stimulation, although IL-2-R expression will be induced on CD2-stimulated thymocytes (5). Single-positive thymocytes, although

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phenotypically indistinguishable from peripheral T cells, apparently require further differentiation to become immunocompetent. The mechanisms via which the relatively small pool of selected, mature thymocytes expand and differentiate into mature, peripheral T lymphocytes is presently unknown.

Previous studies have shown that anti-CD2 antibodies, 9-1 and 9.6, trigger peripheral T cell proliferation that can be augmented by addition of anti-CD3 antibodies (8). The T cell lineage-specific CD28 molecules are also involved in T cell activation in combination with PMA (9, 10). Based on previous work that anti-CD3 and anti-CD28 independently potentiates CD2-mediated T cell activation (11), we found it of interest to study the effects of these T cell mitogenic antibody combinations on thymocytes.

The present study demonstrated that mitogenic combinations of anti-CD2 antibodies together with an anti-CD28 antibody triggers vigorous mature thymocyte proliferation. This cell proliferation was mediated via IL-2 production by autocrine stimulation of IL-2-R. It was observed that only the thymocytes with mature phenotype were able to secrete IL-2 in response to these stimuli. The CD28 molecule (Tp44, 9.3 or Kolt-2) has previously been implicated in an antigen-independent pathway for mature T cell activation (12, 13). These findings together with our data would suggest that the CD2 and CD28 molecules are involved in controlling the expansion and maturation of terminally differentiated thymocytes, and may also regulate self-renewal of the peripheral mature T cell pool.

Materials and Methods

Cell Preparation. Thymic tissue was obtained from children (1 d to 8 yr old) undergoing cardiovascular surgery. Thymocytes were separated, purified, and frozen as previously described (14), or used fresh in the experiments. Thymocyte subpopulations were prepared by the panning method or by cell sorting. Thymocytes, suspended in RPMI 1640 with 0.25% human serum albumin (Armour Pharmaceutical Co., Kankakee, IL) and 25 mM HEPES (Sigma Chemical Co., St. Louis, MO), were reacted with a mixture of anti-CD4 (Leu-3a) and anti-CD8 (Leu-2a) or with anti-CD1 (NA1/34) for isolation of double-negative cells and CD1⁻ or CD1⁺ thymocytes, respectively. After incubating 30 min at 4°C, the cells were washed and either labeled with goat anti-mouse Ig FITC-F(ab)₂ or used for panning. The labeled cells were sorted using a FACS (Coulter EPICS 753; Coulter Electronics Inc., Hialeah, FL). The purity of negatively selected thymocyte subpopulations was assessed by indirect immunofluorescence assays using appropriate mAbs and was always <3% contaminating cells. The CD1⁺ thymocytes were >90% CD1⁺.

mAbs. Anti-CD2 mAbs 9-1 (IgG3), 9.6 (IgG2a), and anti-CD28 mAb 9.3 (IgG2a) were used at a final concentration of 5 µg/ml unless otherwise indicated. mAb 9.3 and 9.6 were gifts from Dr. John A. Hansen, Fred Hutchinson Cancer Research Center, Seattle, WA; the mitogenic anti-CD3 mAb SP34 (IgG2a) was a gift from Dr. Cox Terhorst, Dana Farber Cancer Institute, Boston, MA. The anti-CD25, Tac was purchased from Becton Dickinson & Co. (Mountain View, CA), and the anti-CD25 (T25E) was locally produced at Sloan-Kettering Institute (New York, NY).

Proliferation Assay. Thymocyte proliferation assays were performed as previously described (8, 14). Briefly, thymocytes (10⁵) were cultured in a 96-well plate in 0.2 ml of RPMI 1640 supplemented with 15% human A serum (Plasma Alliance, Inc., Knoxville, TN) for 5 d. [³H]Thymidine (0.5 µCi/well) was added during the final overnight culture. Anti-CD2 antibodies at 5 µg/ml, anti-CD28 (9.3 or KOLT-2) at 5 µg/ml or 1:5,000 dilution of ascites unless otherwise indicated, and anti-CD3 (SP34) at 1:10,000 dilution of ascites were used in the cultures for thymocyte stimulation. Purified human monocyte IL-1 from Dr. Charles Dinarello (Tufts University School of Medicine, Boston, MA) was added at a final concentra-

tion of 5 U/ml and rIL-2 (Cetus Corp., Emeryville, CA) was used at 10 U/ml. Results represent the mean cpm of triplicate samples, with SEM <10% in the majority of the cases.

Northern Blot Analysis. Dot blot analysis was performed with cytoplasmic mRNA obtained from lysing thymocytes that had been cultured for 30 h at 2×10^6 /ml in the presence of mAbs. Pilot time-course experiments showed that IL-2 transcripts reached a peak at ~30 h of stimulation, whereas IL-2 in the supernatants peaked at 48 h of culture. The thymocytes were lysed in a 4 M guanidine thiocyanate solution and chromosomal DNA was sheared. The mixture was layered on a 5.7 M CsCl cushion and spun at 34,000 rpm for 18 h at 4°C. The RNA was resuspended in distilled water and then ethanol-precipitated and resuspended again in distilled water (15). The extracted RNA was dotted onto the nylon membrane (New England Nuclear, Boston, MA). Each dot contained a total RNA from 50×10^6 thymocytes counted at the start of culture. The hybridization was performed at 42°C in the presence of formamide with a 32 P-labeled IL-2 probe (16), an IL-2-R probe (17), or class I HLA probe (pHLA-B7), and the membrane was washed with 0.015 M NaCl/0.015 M Na Citrate/0.5% NaDoSO₄ at 65°C. The membranes were dehybridized by boiling the membrane for 3 min in 0.0015 M NaCl/0.0015 M Na Citrate/0.01% NaDoSO₄. The results were analyzed on the autoradiogram.

Quantitation of IL-2. Supernatants were collected from thymocyte cultures prepared for Northern blot analysis at 30 and 48 h after stimulation. The cell-free supernatants were tested for IL-2 content by the standard bioassay method using the IL-2-dependent murine cell line CTLL. The CTLL line in log phase (4,000 cells/well) was plated in a 96-well flat-bottomed microtiter plate (200 µl). Test samples were added in triplicate to the microtiter plate. After 24 h of culture, the cells were pulsed with 0.5 µCi [3 H]thymidine for the last 4 h of culture and harvested onto filter strips. Results were expressed with 1 U of IL-2 calculated from IL-2 standards.

Immunofluorescence (IF)¹ Staining and Flow Cytometry. Cells were reacted with mAbs for 15 min and washed twice with IF buffer (PBS containing 1% BSA and 0.02% sodium azide). The washed cells were then incubated with goat anti-mouse IgG, F(ab)₂ FITC (Cappel Laboratories, Cochranville, PA) for an additional 15 min and washed with IF buffer. All IF staining procedures were performed on ice. For direct immunofluorescence, cells were directly stained with FITC- or phycoerythrin-conjugated mAbs. The stained cells were resuspended in PBS and were analyzed by FACS.

Results

Activation of Thymocytes Via CD2 and CD28 Triggering. We have investigated the ability of mAbs to CD28, CD2, and CD3 to induce thymocyte proliferation. Fig. 1 shows that unfractionated thymocytes were unresponsive to mitogenic combinations of anti-CD2 (9-1 plus 9.6) antibodies. A vigorous thymocyte proliferation was, however, obtained in a dose-dependent manner with anti-CD28 (9.3), but not with anti-CD3 (SP34) when costimulated with the anti-CD2 combination. Optimal thymocyte proliferation was obtained at 1 µg/ml of 9.3 antibody with 5 µg/ml of 9-1 and 9.6 antibodies. No differences in responses were observed within a range between 1 and 10 µg/ml of 9.3. These findings are in contrast to the results obtained from peripheral mature T cells that proliferate in vitro in response to anti-CD2 (9-1 plus 9.6) alone as well as to the combination of anti-CD2 plus and anti-CD3 (8).

To determine the proliferative potential of the thymocytes triggered by these mitogenic antibody combinations, macrophage-derived IL-1 and rIL-2 were added to unfractionated thymocyte cultures (Table I). Neither exogenous IL-1 nor exogenous IL-2 affected thymocytes cultured with 9-1, 9.6, or 9.3 alone, nor with 9-1 plus 9.3. The anti-CD2 combination 9-1 plus 9.6 could induce IL-2 responsiveness, but not

¹ Abbreviation used in this paper: IF, immunofluorescence.

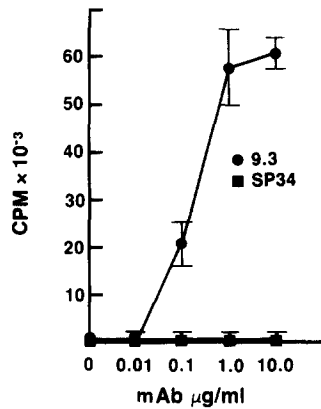


FIGURE 1. Dose response of anti-CD28 (9-3) and anti-CD3 (SP34) for thymocyte proliferation in the presence of optimal concentration of anti-CD2 (9-1 plus 9.6) antibodies. Unfractionated thymocytes were stimulated with different concentrations of 9.3 and SP34 as indicated in the presence of a mixture of 5 µg/ml each of 9-1 and 9.6. Thymidine uptake was measured after 96 h of cell culture as described in Materials and Methods.

IL-1 responsiveness. Interestingly, addition of IL-1 to the anti-CD28 (9.3) plus anti-CD2 (9-1 plus 9.6) combination augmented thymocyte proliferation as much as the addition of exogenous IL-2 to the culture (Table I).

CD28/CD2 Triggering of Thymocytes Induces IL-2 and IL-2-R Expression. Previous studies have shown that certain subsets of thymocytes or mature T cells can proliferate in vitro without IL-2-R or IL-2 (18). To determine whether the IL-2-IL-2-R interaction regulates thymocyte proliferation triggered by the mitogenic 9-1/9.6/9.3 combination, we have performed IL-2 inhibition assays. Two mAbs (Tac and T25E) directed against IL-2-R completely blocked thymocyte proliferation induced by the mitogenic antibody combination (Fig. 2). This result indicated that the thymocyte proliferation induced by anti-CD2 plus anti-CD28 was IL-2 dependent. This result was further substantiated by Northern blot analysis for IL-2 and IL-2-R gene expression in thymocytes. Thymocytes were cultured for 30 h in the presence of various combinations of mAbs as indicated in Fig. 3, and the isolated mRNA was assayed for IL-2 and IL-2-R transcripts.

TABLE I
Proliferation Response of the Unfractionated Thymocytes to the Anti-CD2 and Anti-CD28 in the Presence and Absence of Exogenous IL-1 and IL-2

Stimulators			Medium	IL-1	IL-2
9-1	9.6	9.3			
			<i>cpm × 10⁻³/10⁵ cells</i>		
-	-	-	0.1	0.3	1.0
-	-	+	0.2	0.2	1.4
+	-	-	0.3	0.3	1.6
+	-	+	0.6	0.2	2.8
-	+	-	0.1	0.2	1.4
-	+	+	0.1	0.3	2.2
+	+	-	0.1	0.3	37.1
+	+	+	93.1	212.6	212.2

One of at least five experiments.

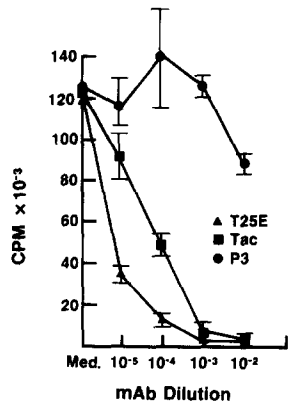


FIGURE 2. IL-2 inhibition assay for thymocyte proliferation induced by the CD2 and CD28-mediated activation using anti-IL-2-R antibodies. Thymocytes were stimulated with a mitogenic combination of mAbs (9-1/9.6/9.3) in the presence of various dilution of anti-IL-2-R, Tac, and T25E. Mouse ascitic fluid P3 with no specificity was used as a negative control.

IL-2-R transcripts were detected in thymocytes cultured with an anti-CD2 combination (9-1 plus 9.6). Addition of anti-CD28 (9.3) induced IL-2 as well as augmenting expression of IL-2-R messages suggesting that the secreted IL-2 may up-regulate its own receptor on thymocytes. Alternatively, the synergistic stimulation via CD2 and CD28 may have augmenting effects on IL-2-R induction. As expected

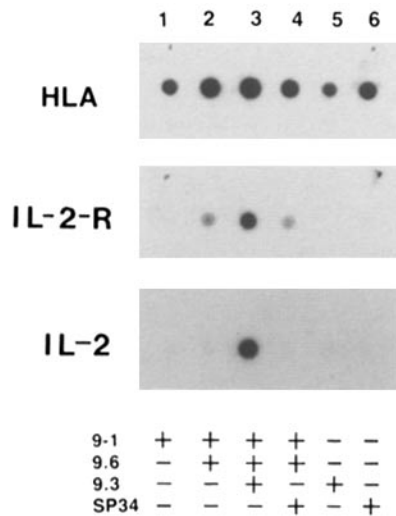
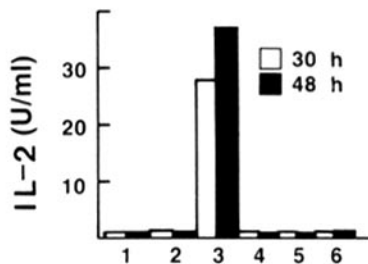


FIGURE 3. Northern dot blot analysis for induction of the IL-2 and IL-2-R transcript and bioassay for IL-2 measurement. Thymocytes were cultured in the presence of various combinations of mAbs as indicated. After 30 h of cell cultures, RNA was isolated, blotted (50×10^6 cells/dot), and hybridized with DNA probes as described in Materials and Methods. A portion of the cells was cultured further to 48 h, and the supernatants were collected. IL-2 released in the supernatants was bioassayed using IL-2-dependent mouse cell line CTLL.



from the results obtained from proliferative assays, anti-CD28 alone or anti-CD3 (SP34) alone had no effect on the expression of IL-2-R transcripts as shown in Fig. 3. As predicted from the above functional studies, 9-1 plus 9.6 did not induce the expression of IL-2 transcripts. In contrast to the results obtained from peripheral mature T cells, addition of anti-CD3 to the anti-CD2 stimulation did not have any effect on IL-2 induction. CD2-mediated IL-2-R induction without IL-2 secretion by thymocytes is consistent with previous observations (5). Stimulation by anti-CD2 (9-1 plus 9.6) and anti-CD28 (9.3) induced IL-2 transcripts in thymocytes, which is consistent with the proliferative in vitro results shown above. A considerable amount of secreted IL-2 was detected in the culture supernatants when thymocytes were activated by the anti-CD2 and anti-CD28 as shown in Fig. 3. None of the other antibody combinations had any effect on IL-2 transcript induction.

CD2/CD28 Triggering is Selective for the CD1⁻, Mature Thymocyte Pool. Recent studies have shown that the immature CD4⁻8⁻ thymocytes have the capacity to secrete IL-2 and to express IL-2-R resulting in proliferation (6). We have examined the double-negative thymocytes for their proliferative responses mediated via the CD2 and CD28 activation pathway. The CD4⁻8⁻ thymocytes were negatively selected by a cell sorter after staining the freshly thawed thymocytes with a mixture of Leu-2a (anti-CD8) and Leu-3a (anti-CD4) antibodies. None of the antibody combinations were able to trigger the CD4⁻8⁻ cells, while unfractionated thymocytes responded as expected (Table II). Double-negative thymocytes obtained by panning procedure gave similar results (data not shown). However, the mitogenic combination 9-1/9.6/9.3 induced a vigorous proliferation of the cells positively selected for CD4⁺ and CD8⁺ expression (data not shown). However, the data were difficult to interpret since the positively selected cells also contained both double-positive and single-positive cells. Furthermore, antibodies bound to thymocytes could potentially exert either a positive or negative effect on cell membranes.

To overcome these difficulties, the double-positive thymocyte population was isolated by cell sorting using the CD1 cell surface marker. CD1 molecules are predominantly expressed on immature thymocytes, particular by intermediate CD4⁺8⁺

TABLE II
Activation of Highly Purified Double-negative (CD4⁻8⁻)
Thymocytes by Anti-CD2 and Anti-CD28

Stimulators	Exp. 1	Exp. 2
	<i>cpm × 10⁻³/10⁵ cells*</i>	
Medium control	0.2	0.6
9-1 + 9.3	0.2	0.4
9.6 + 9.3	0.3	0.2
9-1 + 9.6	0.1	0.5
9-1 + 9.6 + 9.3	0.2	1.0
Total thymocytes	51.8 [†]	75.7 [‡]

* The double-negative thymocytes were negatively selected by FACS after staining with a mixture of anti-CD4 (Leu-3a) and anti-CD8 (Leu-2a) mAbs.

† cpm obtained by stimulation of 9-1 + 9.6 + 9.3.

TABLE III
Proliferation Responses for the CD1⁺ and CD1⁻ Thymocytes
Triggered by Anti-CD2 and Anti-CD28

Culture conditions	Exp. 1	Exp. 2
	<i>cpm</i> × 10 ⁻³ /10 ⁵ cells	
Total thymocytes		
Medium control	0.1	0.1
9-1 + 9.6	0.1	0.3
9-1 + 9.6 + 9.3	36.0	116.5
CD1 ⁺ thymocytes*		
Medium control	<0.1	0.1
9-1 + 9.6	<0.1	0.1
9-1 + 9.6 + 9.3	0.3	15.4
CD1 ⁻ thymocytes*		
Medium control	<0.1	0.1
9-1 + 9.6	<0.1	0.2
9-1 + 9.6 + 9.3	52.1	78.8

* The CD1⁺ and CD1⁻ thymocytes were sorted by FACS after staining with anti-CD1 mAb NA1/34.

cells, and CD1 expression is lost on the mature single-positive cells (4). Table III shows the proliferative response of the CD1⁺ and CD1⁻ subsets when stimulated with the anti-CD2 and anti-CD28 antibodies. The subpopulations were sorted after fluorescence staining with the anti-CD1 (NA1/34) antibody. The 9-1/9.6/9.3 stimulation consistently triggered proliferation of the CD1⁻ cells, but a greatly reduced proliferative response was obtained for the CD1⁺ cells (Table III). These results, obtained from the CD4⁻8⁻ fraction together with data from the CD1⁺ and CD1⁻ cells, indicated that only the mature single-positive thymocytes have the capacity to proliferate via the CD2 and CD28 activation pathway.

The *in vitro* proliferative studies demonstrated that the anti-CD2 plus anti-CD28 responsive thymocytes are the mature thymocytes. These results were consistent with data obtained by staining thymus sections with mAb 9.3 (Fig. 4) and cytofluorographic analysis of thymocytes (Fig. 5). The anti-CD28 stained brightly the medullary section, which contains mature thymocytes (Fig. 4 B), with rare cells in the thymus cortex reacting with the CD28 antibody (Fig. 4 A). FACS analysis of unfractionated cryopreserved thymocytes showed that 55% of thymocytes were positive for anti-CD28 (9.3) with a relatively low intensity, whereas 70% were stained with anti-CD3 (Leu-4). This high percentage of CD28⁺ thymocytes (Fig. 5 a) indicated that the CD28⁺ subset survived preferentially from the cryopreservation and thawing process, since the percentage profile for CD28 antigens on fresh thymocytes was 10–20% in several experiments. Two-color analysis of thymocytes showed that the majority of CD28⁺ cells were also CD3⁺ cells: 39% were CD28⁺/CD3⁺, 24% CD28⁻/CD3⁺, 6% CD28⁺/CD3⁻, and 31% CD28⁻/CD3⁻ (data not shown). It remains to be determined if there are differences in the capacity of the CD28⁺/CD3⁻ and CD28⁺/CD3⁺ thymocytes to respond to costimulation with anti-CD2 and anti-CD28.

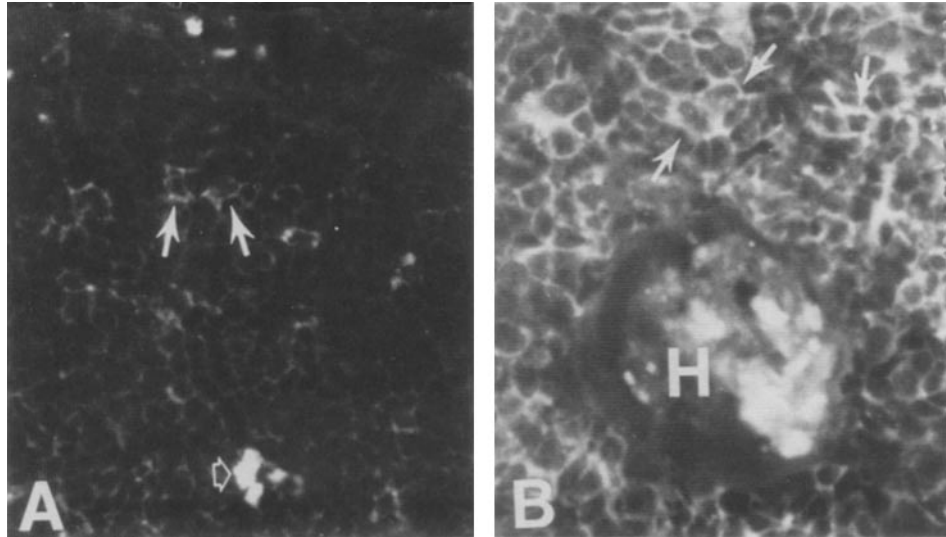


FIGURE 4. Human thymus cross section stained with anti-CD28 (9.3). (A) The reactivity of mAb 9.3 with thymocytes in the thymic cortical zone. Solid arrows point to 9.3⁺ thymocytes, and open arrows denote autofluorescent (9.3⁻) pigment. (B) The reactivity of 9.3 with thymocytes in the thymic medulla. H identifies a Hassall's body region. Most medullary thymocytes are 9.3⁺, whereas few cortical thymocytes are 9.3⁺ (× 400).

Discussion

Our results demonstrated that CD1⁻ mature thymocytes can proliferate *in vitro* in an autocrine fashion when triggered by a combination of CD2 and CD28 mAbs in the absence of exogenous cytokines. We used two anti-CD2 antibodies (9-1 plus

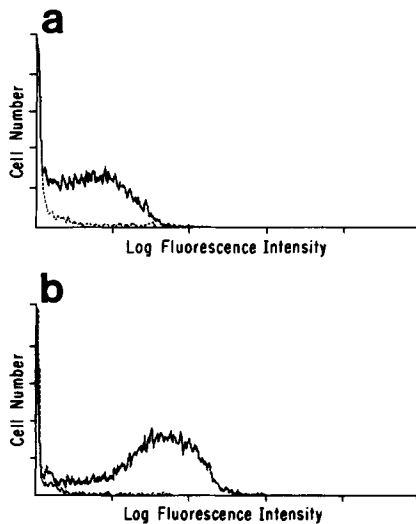


FIGURE 5. Reactivity patterns for CD28 and CD3 on unfractionated thymocytes by FACS. Thymocytes were indirectly stained with 9.3 (anti-CD28) (a) and Leu-4 (anti-CD3) (b). In this experiment, 55% of thymocytes reacted with mAb 9.3, while 70% reacted with mAb Leu-4.

9.6), which can trigger peripheral mature T cell proliferation in the absence of macrophages (8), together with an anti-CD28 antibody, which is also mitogenic for T cells in combination with PMA (9, 10). The anti-CD2 combination alone or anti-CD28 alone rendered no effect on thymocyte proliferation. Addition of anti-CD3 to the anti-CD2 combination, which normally augments proliferation of mature peripheral T cells (8), did not drive thymocytes to proliferate. We have found that the proliferative thymocyte response to the anti-CD2 plus anti-CD28 antibody combination was confined to the thymocyte subset of mature cells with the phenotype of CD3⁺CD1⁻/CD4⁺ or CD8⁺. These results indicate that, in contrast to peripheral T cells, thymocytes are unable to respond in the absence of exogenous cytokines to triggering signals via CD2 or CD3/Ti pathways in spite of their mature phenotype. However, as previously reported (5), thymocytes treated with the anti-CD2 combination were able to proliferate in the presence of exogenous IL-2, indicating that the activation block in thymocytes is in the production of endogenous IL-2. Previous studies have also shown that stimulation of the CD3/Ti complex on thymocytes by anti-CD3 inhibit IL-2 gene activation, which can be induced by the mitogenic anti-CD2 antibody combination plus PMA (19). Taken together these results would imply that CD3⁺ mature thymocytes are functionally distinct from peripheral mature T lymphocytes despite their mature phenotype. The CD3/Ti interactions with self MHC products in the thymus could thereby function as a down-regulation signal for the selection against autoreactive T cell precursors as has recently been suggested (19).

Our study demonstrates that the majority of the CD28⁺ thymocytes are CD3⁺. These CD28⁺/CD3⁺ cells are localized primarily within the medullary compartment of the thymus (Fig. 4), where mature thymocytes reside. Growing evidence indicates that proliferation is associated with maturation of thymocytes, and IL-2 is the likely rate-limiting factor for the growth of some thymocyte subsets (20). Since virtually all cells in the T cell lineage including all stages of thymocytes express the CD2 antigen, it is tempting to speculate that activation of the CD2 antigen plays an important role in intrathymic mediation of thymocyte proliferation. Our present study provides evidence that the combination of activation signals to CD2 and CD28 could account for such cell proliferation signals for the mature stage III thymocytes.

The CD28 antigen is a T cell lineage-specific cell surface molecule first defined by mAb 9.3 (21). Previous studies have shown that activation of the CD28 molecule has a synergistic effect on PMA-induced activation of protein kinase C, resulting in IL-2 production and IL-2-R expression causing vigorous T cell proliferation (9, 10). The CD28-mediated T cell activation is dependent upon accessory cells (12, 13) as is also the case for T cells activated via CD3/Ti (22). Our study demonstrates that macrophage-derived IL-1 augments thymocyte proliferation triggered by costimulation with anti-CD28 and anti-CD2 (9-1 plus 9.6). IL-1 did not have any effect on thymocytes stimulated with the mitogenic anti-CD2 antibody combination alone. These results would indicate that IL-1 plays a role in CD28-triggered thymocyte proliferation. This is consistent with data demonstrating that IL-1 acts as a cofactor for thymocyte activation (23).

A functional interaction between the CD3/Ti complex and CD2 suggests that physiologic activation of CD3/Ti involves a cascade of events in which the CD2 molecule plays a central role (8, 24). Anti-CD28 triggers mature thymocyte proliferation when

added to mitogenic anti-CD2 antibodies as shown in the present study. Anti-CD28 also potentiate peripheral T cell proliferation induced by anti-CD2 stimulation (11). These findings suggest that there are two distinct pathways for T cell activation: an antigen-specific activation pathway involving CD3/Ti and CD2, and an antigen-independent pathway involving CD28 and CD2. Activation of the CD3/Ti complex on mature thymocyte results in down-regulation of IL-2 gene expression (19), providing a selective advantage for CD28/CD2 activation of these cells. This observation suggests that both of these activation pathways are involved in selection and expansion of thymocytes as well as regulation of the peripheral T cell pool. Cellular regulation of these two T cell activation pathways is probably achieved by second intracellular messengers such as cAMP and cGMP. It has been previously shown that CD28-mediated activation elevates intracellular cGMP levels (25, 26), whereas the CD3/Ti-mediated T cell activation increases cAMP levels (27).

Recent studies have demonstrated that the CD28 molecule has physical and functional characteristics that are very similar to the antigen-specific TCR molecule (10, 12); both belong to the Ig super gene family (28, 29). These findings could reflect an evolutionary relationship between the antigen-specific TCR (Ti) and the CD28 molecule. Both of these molecules could have maintained a common functional interaction with the CD2 molecule resulting in identical functional end effects, i.e., T cell activation and cell proliferation mediated via IL-2 production and IL-2-R gene expression. The Ti molecule evolved into a diverse set of antigen-specific receptor molecules, while the CD28 molecule became the receptor for a presently unidentified ligand responsible for recruitment of mature T cells.

The CD28 molecule is expressed on ~95% of peripheral CD4⁺ T cells and on ~50% of peripheral CD8⁺ T cells (30). These CD28⁺ mature, peripheral T cells could provide a peripheral T cell compartment from which fully differentiated mature T cells can be recruited when required. The CD28⁻ peripheral T cells would in this model contain the fully differentiated antigen-specific T cell compartment, which can only be activated and expanded via CD3/Ti antigen-specific activation.

The present study demonstrates a novel activation pathway for mature thymocytes involving CD28 and CD2. This activation pathway is also functional in peripheral mature T cells expressing CD28. The data presented support a model for maturation and expansion of mature thymocytes in their transition from thymocytes to the periphery. The CD28/CD2 pathway is antigen independent and may therefore function by regulating and maintaining the peripheral T cell pool. It is speculated that the CD28/CD2 antigen-independent T cell activation pathway, and the CD3/Ti/CD2 antigen-specific pathway are the two major systems for regulation and expansion of mature T lymphocytes.

Summary

Prior studies have shown that thymocytes, unlike peripheral T cells, do not proliferate in response to mitogenic combinations of anti-CD2 mAbs. The present study demonstrated that stimulation by a mitogenic anti-CD2 combination (9-1 plus 9.6) with anti-CD28 induced vigorous thymocyte proliferation in the absence of exogenous IL-2. This thymocyte proliferation was IL-2 dependent as shown by the complete inhibition using anti-IL-2-R mAbs. Induction of IL-2-R transcripts was detected in thymocytes stimulated by the anti-CD2 antibody combination alone or the anti-

CD2 combination plus anti-CD28 antibody. However, induction of IL-2 transcripts was observed only in thymocytes triggered jointly by the anti-CD2 combination plus anti-CD28 antibodies. The double-negative ($CD4^- 8^-$) or $CD1^+$ thymocytes isolated by sorting or by panning were unresponsive to CD2/CD28 triggering. The same mitogenic signal could induce vigorous proliferation of thymocytes with a mature phenotype, i.e., $CD3^+ CD4^+$ or $CD3^+ CD8^+$ thymocytes. Immunofluorescence studies demonstrated that the majority of $CD3^+$ thymocytes were $CD28^+$, and most of the $CD28^+$ cells were located in the medullary compartment of thymus. These results indicated that the T cell lineage surface molecules CD28 and CD2 are involved in the regulation of expansion and further differentiation of mature thymocytes.

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