0022-1767/88/1419-2980$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 141, 2980–2985, No. 9, November 1, 1988
Printed in U.S.A.

PURIFIED LYMPHOCYTE FUNCTION-ASSOCIATED ANTIGEN-3 (LFA-3) ACTIVATES HUMAN THYMOCYTES VIA THE CD2 PATHWAY[1]

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Defining the cellular and molecular mechanisms of interaction of developing thymocytes with nonlymphoid cells of the thymic microenvironment is critical for understanding normal thymus function. We have previously shown that the CD2/LFA-3 adhesion pathway is important in the interaction of thymocytes with a variety of LFA-3⁺ nonlymphoid thymic microenvironment cell types. Moreover, T cell activation via the CD2 (alternative, Ag independent) pathway is considered an important mechanism for intrathymic T cell proliferation. To study the relevance of CD2/LFA-3 interactions to human thymocyte activation, we have used purified LFA-3 Ag in several in vitro assays of thymocyte proliferation. Whereas LFA-3 Ag alone did not induce thymocyte proliferation, LFA-3 Ag in combination with the anti-CD2 antibody, CD2.1, and rIL-2 induced marked thymocyte proliferation. Additionally, the anti-CD28 antibody, Kolt2, could substitute for rIL-2, resulting in thymocyte activation induced by LFA-3 Ag in combination with antibodies CD2.1 and Kolt2. In both triggering systems, LFA-3 induced thymocyte activation was dependent upon the concentration of LFA-3 Ag. LFA-3 Ag-dependent thymocyte activation was directed primarily toward CD1⁻, mature thymocytes. Finally, intact SRBC that express the sheep homolog of LFA-3, T11TS, in combination with antibody CD2.1 and rIL-2 could also induce thymocyte activation. These data suggest that interaction of LFA-3 molecules with thymocyte CD2 molecules may provide a component of the stimulus for normal intrathymic thymocyte activation leading to thymocyte proliferation.

The CD2[3] T cell molecule is present on all thymocytes and peripheral blood T cells, and is acquired on fetal T cells at 8 wk of gestation soon after T cell precursors enter the epithelial thymic rudiment (1, 2). In addition to serving as the receptor via which T cells bind SRBC, the CD2 molecule functions in an Ag-independent alternative pathway of T cell activation (3, 4). This pathway of T cell activation was initially observed in peripheral blood T cells in vitro in response to combinations of CD2 mAb that bind to distinct epitopes ($T11_2$, $T11_3$) of the CD2 molecule (4–7), The observation that thymocytes could be activated to express IL-2R in response to specific combinations of CD2 mAb suggested that the CD2 molecule may be an important structure by which thymocytes are activated to proliferate intrathymically (8). Recently studies of the molecules involved in cytolytic T cell (CTL) target cell interactions (9–11), thymic epithelial cell-thymocyte interactions (12, 13) and SRBC-T cell interactions (14–17), have suggested that LFA-3 molecules on thymic epithelial cells, CTL target cells, and SRBC interact with T cell CD2 molecules (18). A critical question with regard to study of the role of CD2 triggering of thymocytes in driving intrathymic T cell maturation is whether the binding of LFA-3 molecules to thymocyte CD2 molecules indeed leads to thymocyte proliferation. In this paper, we show that affinity purified LFA-3 Ag from human E and as well, intact SRBC, can provide activation signals to thymocytes via thymocyte CD2 molecules. However, LFA-3 molecule binding to thymocyte CD2 molecules was not by itself sufficient for CD2 triggering of thymocytes. Rather, CD2 triggering of thymocytes by soluble LFA-3 Ag or by intact SRBC required the simultaneous addition of an anti-CD2 antibody (CD2.1 or 9-1) as well as the addition of exogenous IL-2.

MATERIALS AND METHODS

mAb. mAb TS2/9 was used as previously described (10). Antibodies 35.1 (19), 9-1 (6), NA1/34 (20), anti-TAC (21) DMS-1 (22), and T3/2T8-2F4 were the gifts of Drs. John Hansen, Soo Young Yang and Bo DuPont, A.J. McMichael, Thomas Waldmann, Kendall Smith, and Ellis Reinherz, respectively. Antibodies Kolt2 [CD28] (23) and CD2.1 [CD2] (5) from the laboratories of Drs. Kimitaka Sagawa and Daniel Olive, respectively, were obtained from the T cell panel of the Second International Workshop on Leukocyte Differentiation Antigens (24). P3X63Ag8 ascites was used as a negative control in all experiments.

LFA-3 Ag purification. LFA-3 Ag was purified from Triton X-100 lysates of human E by immunoaffinity chromatography on TS2/9-Sepharose CL-4B (Pharmacia, Piskataway, NJ) (5). After extensive washing of the immunoaffinity column, LFA-3 was eluted at pH 3 in the presence of 1% octylglucoside (Cal-Biochem Behring, San Diego, CA) detergent. Aggregated LFA-3 was prepared by three cycles of ultrafiltration by using a Centricon 30 apparatus (Amicon, Danvers, MA) by adding 2 ml of PBS and reducing the volume to 50 μl with each cycle. The final LFA-3 Ag preparation contained less than

Received for publication March 15, 1988.
Accepted for publication August 2, 1988.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] This work was supported in part by Grants CA28936, CA31798, A123308, AR39162, and AR34808 from the National Institutes of Health. K. H. S. is a Scholar of the Leukemia Society of America. S. M. D. is a recipient of a Clinician Scientist Award from the American Heart Association.
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[3] Abbreviations used in this paper: CD, cluster of differentiation; LFA-3, lymphocyte function associated Ag-3; Ti, T cell receptor for antigen; T11TS, T11 target structure.

0.01% octylglucoside.

Cell preparation and proliferation assays. Thymic tissue was obtained from normal subjects undergoing median sternotomy incision and corrective cardiovascular surgery. Subjects ranged in age from 10 days to 6 yr. Thymocytes were purified, slow frozen, and stored in liquid nitrogen as described (25). Thymocytes were thawed and proliferation assays established by modification of previously described methods (13, 25). Briefly, 1×10^5 thymocytes in 0.2 ml RPMI 1640 (GIBCO Laboratories, Grand Island, NY) + 15% human A serum (Plasma Alliance Inc., Knoxville, TN) were cultured in 96-well round-bottom plates (Costar, Cambridge, MA). mAb 9-1, CD2.1, 35.1, and Kolt2 were added to thymocyte cultures at 1 μg/ml, 1/1000 dilution of ascites, 1/5000 dilution of ascites, and 1/5000 dilution of ascites, respectively. For addition of LFA-3 Ag to thymocyte cultures, LFA-3 Ag was diluted in human A serum and added to cultures at a final concentration of 250 ng/ml or as described.

Cell separations to prepare $CD1^+$ and $CD1^-$ thymocytes were performed by treatment of thymocytes with antibody NA1/34 (20) followed by panning on immunoglobulin coated polystyrene dishes (26, 27). The $CD1^+$ thymocyte suspensions were 83% $CD1^+$, whereas the $CD1^-$ thymocyte suspensions were 11% $CD1^+$.

In certain experiments, thymocytes were separated by preparative flow cytometry by using an EPICS 753 flow cytometer (Coulter Electronics, Hialeah, FL). After incubation with antibody NA1/34 at 4°C, cells were washed twice with RPMI 1640, incubated with fluorescein-conjugated goat anti-mouse Ig (Kirkegaard & Perry Laboratories, Gaithersburg, MD), washed twice, and sorted. The $CD1^+$ population recovered after preparative flow cytometry was greater than 95% $CD1^+$. Preliminary experiments with antibody NA1/34 revealed that addition of antibody to the cultures or staining of cells before culture did not affect thymocyte proliferative responses to LFA-3 Ag.

In certain experiments, rIL-2, the gift of Cetus Corporation (Emeryville, CA), was added at a concentration of 1 U/ml or as described. After a 4-day incubation at 37°C in humidified 5% CO_2 environment, 0.4 μCi of [^{3}H]thymidine (New England Nuclear, Boston, MA) was added to each well and incubated 4 h. Wells were harvested onto glass fiber filters with a Mash II harvester (Whittaker MA Bioproducts, Walkersville, MD). Isotope incorporation was determined by scintillation counting in a Tri-Carb liquid scintillation counter (Packard Instrument Co., Downers Grove, IL). Results are expressed as cpm per 10^6 thymocytes placed in culture. Data were compared with the use of Student's *t*-test.

Antibody TS2/9 (anti-LFA-3) or anti-TAC (anti-IL2R) was added to certain thymocyte cultures at 1/200 dilution or 1/500 dilution of ascites, respectively. Antibody DMS-1 (anti-IL-2) was added to thymocyte cultures at 100 μg/ml. Antibody T3/2T8-2F4 was added to thymocyte cultures at 1/1000 dilution of ascites.

SRBC were washed in PBS (pH 7.0) and added to thymocytes in the presence of 9-1, CD2.1, or 35.1 antibodies at a concentration of 3×10^6 SRBC per 1×10^5 thymocytes.

RESULTS

LFA-3 Ag-mediated thymocyte activation in the presence of IL-2. LFA-3 Ag when added alone directly to thymocyte cultures induced little thymocyte activation as measured by [^{3}H]thymidine incorporation (Table I). Addition of LFA-3 Ag (250 ng/ml) to thymocytes in combination with any of the anti-CD2 antibodies 35.1, CD2.1, or 9-1 also failed to induce thymocyte activation (Table I). Addition of 1 U/ml of rIL-2 yielded a slight increase in thymocyte proliferation. However, addition of LFA-3 Ag (250 ng/ml) together with antibody CD2.1 ($T11_2$) and rIL-2 induced significant thymocyte proliferation ($p < 0.025$) (Table I). Additionally, the $T11_3$ anti-CD2 antibody, 9-1, but not the $T11_2$ anti-CD2 antibody, 35.1, also stimulated thymocyte proliferation in combination with LFA-3 Ag and rIL-2, though not to the level of stimulation seen with the anti-CD2 antibody, CD2.1 (Table I). To determine the LFA-3 Ag concentration necessary to maximally induce thymocyte activation, graded amounts of purified LFA-3 Ag were added to thymocyte cultures in the presence of CD2.1 and 5 U/ml of rIL-2. A detectable increase in thymocyte proliferation was observed at an LFA-3 Ag concentration of 50 ng/ml (Fig. 1*A*), with thymocyte ac-

TABLE I

LFA-3 Ag induces human thymocyte proliferation in the presence of rIL-2[a]

Additive to Thymocyte Suspension	[^{3}H]Thymidine Incorporation (cpm) per 10^6 Thymocytes		
	Expt. 1	Expt. 2	Expt. 3
Media	2,000	800	500
CD2.1	7,400	1,200	900
9-1	6,200	700	1,300
35.1	5,100	1,700	1,200
rIL-2	5,100	18,700	7,000
CD2.1 + rIL-2	7,400	13,300	3,800
9-1 + rIL-2	4,800	11,800	4,800
35.1 + rIL-2	4,000	16,600	7,100
LFA-3 Ag	4,800	800	900
+ CD2.1	700	2,100	1,500
+ 9-1	1,500	1,700	700
+ 35.1	800	1,900	1,200
+ rIL-2	3,400	13,700	4,700
+ CD2.1 + rIL-2	29,200	55,100	30,800
+ 9-1 + rIL-2	7,000	34,400	15,700
+ 35.1 + rIL-2	5,300	15,400	10,700

[a] Thymocytes were prepared and cultures initiated as described in *Materials and Methods*. Purified LFA-3 Ag was added at 250 ng/ml final concentration in human A serum. rIL-2 was added at 1 U/ml. Data shown are [^{3}H]thymidine incorporation after 4 days in culture. Results shown are three representative experiments of five performed. The cpm of LFA-3 Ag + CD2.1 + IL-2 were significantly different from LFA-3 Ag + IL-2 ($p < 0.025$). Activation of thymocytes by using 9-1 + LFA-3 Ag + rIL-2 was less pronounced than that seen in experiments with CD2.1 antibody.

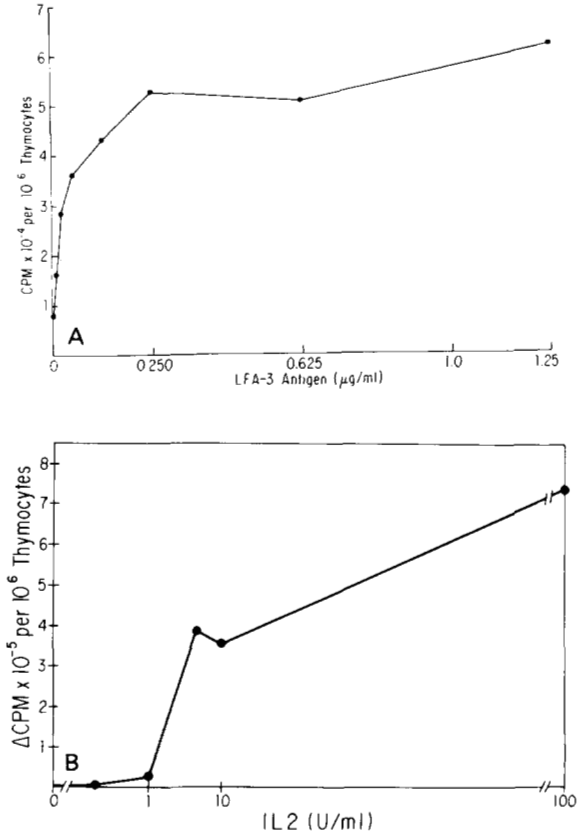

Figure 1. Dose response for LFA-3 Ag and rIL-2-induced thymocyte proliferation. *A*, Thymocytes were cultured with the indicated concentration of LFA-3 Ag in the presence of CD2.1 (1/5000 dilution of ascites) and rIL-2 5 U/ml. Wells were pulsed with [^{3}H]thymidine during the final 4 h of a 4-day culture. Results shown are representative of two experiments performed. *B*, Thymocytes were cultured with the indicated concentration of rIL-2 in the presence of CD2.1 (1/5000 dilution of ascites) and LFA-3 Ag, 0.25 μg/ml. Wells were pulsed with [^{3}H]thymidine during the final 4 h of a 4-day culture. Results are Δ cpm = cpm of culture stimulated with CD2.1 + LFA-3 Ag + rIL-2 − cpm of culture stimulated with rIL-2 only.

tivation reaching a plateau at approximately 250 ng/ml. Figure 1*B* shows the titration of rIL-2 in this system with initial proliferative responses seen at 1 U/ml of rIL-2.

Substitution of anti-CD28 antibody for exogenous rIL-2 in LFA-3 Ag-induced thymocyte activation. CD28 antibodies have been reported to augment peripheral T cell proliferative responses to a variety of stimuli by inducing T cell IL-2 production (28–30). To determine whether LFA-3 Ag could induce thymocyte activation in the absence of rIL-2 but in the presence of anti-CD28 antibody, LFA-3 Ag was added to thymocyte cultures in combination with anti-CD2 (CD2.1) and anti-CD28 (Kolt2) antibodies. As before, LFA-3 Ag alone or in combination with any of the CD2 antibodies effected no thymocyte proliferation (Table II). The combination of anti-CD28 antibody and LFA-3 Ag or of anti-CD28 and anti-CD2 (CD2.1) antibodies also failed to induce thymocyte proliferation. However, the combination of LFA-3 Ag, the anti-CD2 antibody, CD2.1, and the anti-CD28 antibody, Kolt2, stimulated significant thymocyte proliferation ($p < 0.025$). The combination of LFA-3 Ag, anti-CD2 antibody, 35.1, and antibody Kolt2 failed to induce thymocyte proliferation. In contrast to experiments with rIL-2, in the presence of Kolt2, the combination of anti-CD2 antibody 9-1 and LFA-3 Ag activated thymocytes in only two of five experiments (Table II). Figure 2 shows the dose response of LFA-3 Ag in CD2.1 + Kolt2-stimulated thymocyte cultures, with a substantial proliferative response seen with 125 ng/ml of LFA-3 Ag.

Dependence of LFA-3 Ag-induced thymocyte activation on IL-2. To further demonstrate directly the dependence on IL-2 of LFA-3 Ag-induced thymocyte activation, a mAb to the IL-2R (anti-TAC, CD25), was added to thymocyte cultures stimulated with LFA-3 Ag and antibodies CD2.1 and Kolt2 (Fig. 3). Control cultures performed simultaneously contained P3×63 ascites or the anti-LFA-3 antibody, TS2/9. Both anti-TAC and anti-LFA-3 antibodies inhibited LFA-3 Ag-induced thymocyte activation. In addition, the anti-IL-2 mAb DMS-1 (22) also inhibited LFA-3 Ag-induced thymocyte activation by 68 ± 11% ($p < 0.05$) (not shown).

LFA-3 Ag-induced thymocyte activation is inhibited by anti-CD3 antibody. Because the CD2 alternative pathway of mature T cell activation is regulated by the CD3-Ti complex (4), we tested the effect of addition of a nonmitogenic CD3 antibody on LFA-3 Ag-induced thymocyte activation. Addition of antibody to CD3 alone or in combination with rIL-2 induced a slight thymocyte proliferative response (Table III). However, addition of anti-CD3 antibody, T3/2T8-2F4, to LFA-3 Ag-stimulated cultures inhibited 89% of the thymocyte proliferative response to the combination of LFA-3 Ag, CD2.1, and rIL-2 (Table III).

LFA-3 Ag-induced thymocyte activation is directed primarily toward mature thymocytes. The phenotype of responding thymocytes in LFA-3 Ag-stimulated cultures was determined by separation of populations enriched for $CD1^+$ (inner cortical immature) thymocytes and $CD1^-$ (mature) thymocytes. Cultures were initiated with 250 ng LFA-3 Ag, CD2.1 antibody, and rIL-2 (Fig. 4*A*) or the anti-CD28 antibody Kolt2 (Fig. 4*B*). The primary responding cell type in both systems was $CD1^-$ (mature) thymocytes. However, $CD1^+$ thymocytes also proliferated in response

TABLE II

LFA-3 Ag induces thymocyte proliferation in combination with CD2.1 and Kolt2[a]

Additive to Thymocyte Suspension	[^{3}H]Thymidine Incorporation (cpm) per 10^6 Thymocytes	
	Expt. 1	Expt. 2
Media	800	1,700
9-1 + Kolt2	1,800	800
CD2.1 + Kolt2	1,900	600
35.1 + Kolt2	1,100	1,800
LFA-3 Ag	800	2,400
+ 9-1	1,700	4,500
+ CD2.1	2,100	1,800
+ 35.1	1,900	500
+ Kolt2	1,100	900
+ 9-1 + Kolt2	3,500	43,500
+ CD2.1 + Kolt2	127,600	68,700
+ 35.1 + Kolt2	800	900

[a] Thymocytes were prepared and cultures initiated as described in *Materials and Methods*. LFA-3 Ag was added at 250 ng/ml final concentration in human A serum. Data shown are the [^{3}H]thymidine incorporation after 4 days in culture. LFA-3 Ag + CD2.1 + Kolt2 is significantly ($p < 0.025$) greater than LFA-3 Ag + CD2.1. LFA-3 Ag + 9-1 + Kolt2 activated thymocytes in only two of five experiments performed. The experiments shown represent the two patterns since LFA-3 Ag in combination with 9-1 and Kolt2 activated thymocytes in experiment 2 but not in experiment 1.

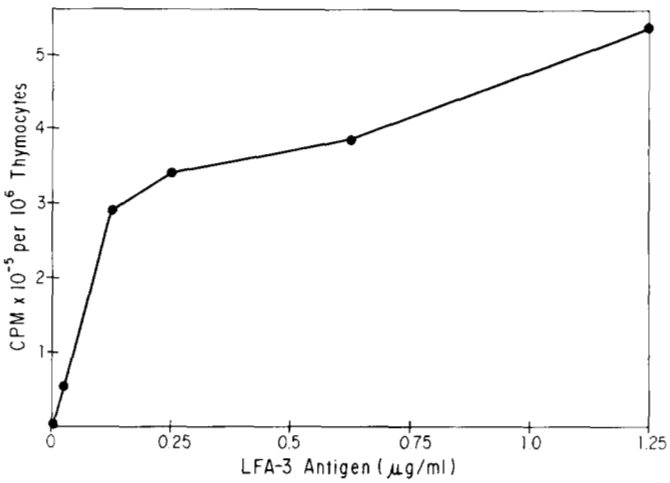

Figure 2. Dose response for LFA-3 Ag-induced thymocyte proliferation. Thymocytes were cultured with the indicated concentrations of LFA-3 Ag in the presence of CD2.1 and Kolt2 antibodies (1/5000 dilutions of ascites). Wells were pulsed with [^{3}H]thymidine during the final 4 h of a 4-day incubation.

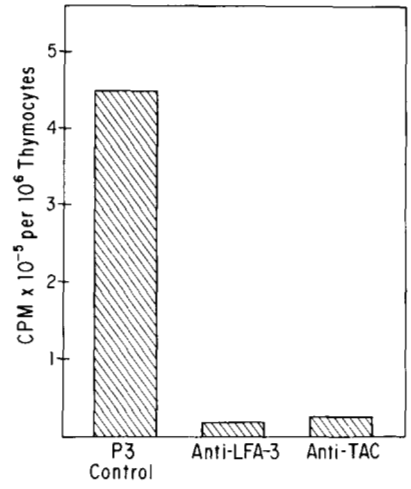

Figure 3. LFA-3 Ag-induced thymocyte activation is inhibited by anti-LFA-3 and anti-TAC antibodies. Thymocytes were cultured in the presence of LFA-3 Ag (250 ng/ml) and CD2.1 and Kolt2 antibodies (1/5000 dilutions of ascites). Antibodies TS2/9, TAC, or P3×63 control were added at a 1/200 dilution of ascites. Wells were pulsed with [^{3}H]thymidine during the final 4 h of a 4-day culture.

TABLE III

Anti-CD3 antibodies inhibit LFA-3 Ag induced thymocyte proliferation[a]

Additive to Thymocyte Suspension	[^{3}H]Thymidine Incorporation (cpm) per 10^6 Thymocytes
Media	1,500
Anti-CD3	15,600
rIL-2	3,600
Anti-CD3 + rIL-2	21,000
LFA-3 Ag + CD2.1 + rIL-2	147,300
Anti-CD3 + LFA-3 Ag + CD2.1 + rIL-2	16,500

[a] Thymocyte cultures were initiated as described in *Materials and Methods*. LFA-3 Ag concentration was 250 ng/ml; rIL-2 was added at 5 U/ml. Anti-CD3 antibody, T3/2T8-2F4, was added at a 1/1000 dilution of ascites. Data are mean [^{3}H]thymidine incorporation after a 4-day culture.

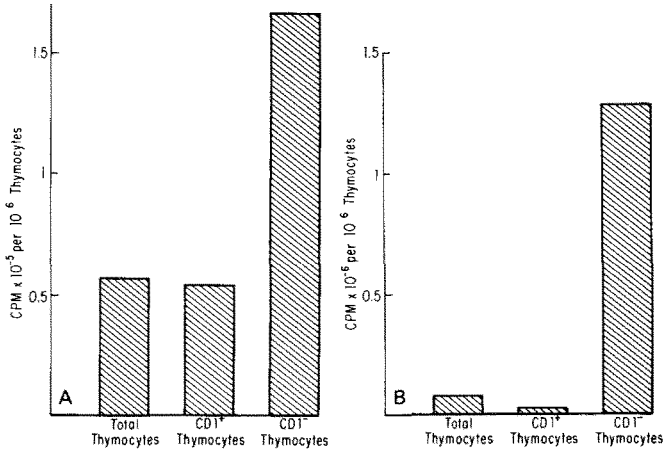

Figure 4. LFA-3 induced thymocyte activation is directed primarily toward mature thymocytes. *A*, Thymocytes were separated into CD1$^+$ and CD1$^-$ subpopulations by panning (26, 27). Thymocyte cultures were initiated in the presence of LFA-3 Ag, 0.25 μg/ml, CD2.1 (1/5000 dilution of ascites) and rIL-2, 5 U/ml. Wells were pulsed with [^{3}H]thymidine during the final 4 h of a 4-day incubation. *B*, Thymocytes were separated into CD1$^+$ and CD1$^-$ subpopulations by panning (26, 27). Thymocyte cultures were initiated in the presence of LFA-3 Ag, 0.25 μg/ml, and antibodies CD2.1 and Kolt2 (1/5000 dilutions of ascites). Wells were pulsed with [^{3}H] thymidine during the final 4 h of a 4-day incubation.

to LFA-3 Ag, antibody CD2.1 and rIL-2 (Fig. 4*A*). To assess further the proliferative response of CD1$^+$ thymocytes, highly purified (>95% CD1$^+$) thymocytes were cultured with LFA-3 Ag, antibody CD2.1, and rIL-2. In two experiments, highly purified CD1$^+$ thymocytes also proliferated to CD2 ligands (LFA-3 Ag + antibody CD2.1) and rIL-2 to the same degree as that shown for enriched CD1$^+$ cells in Figure 4*A*.

SRBC activation of human thymocytes. SRBC binding to human T cells and thymocytes is mediated via the CD2 Ag on T cells and the sheep homolog of human LFA-3 Ag on SRBC termed T11 target structure (T11TS) (14–17). To determine whether intact SRBC could act as a functional ligand for thymocytes in the same manner as purified human E LFA-3 Ag, we tested SRBC in these same CD2-dependent assays of thymocyte activation in which LFA-3 Ag activated thymocytes. As with purified LFA-3 Ag, SRBC either alone or in combination with an anti-CD2 antibody failed to induce thymocyte proliferation (Table 4). Exogenous rIL-2 (1 U/ml) induced minimal thymocyte activation alone or in combination with SRBC. However, the combination of SRBC, rIL-2, and anti-CD2 antibody CD2.1 or antibody 9-1 induced significant thymocyte proliferation. Substitution of the $T11_2$ anti-CD2 antibody 35.1 for CD2.1 or 9-1 in this system resulted in

TABLE IV

SRBC and anti-CD2 antibodies 9-1 or CD2.1 induce thymocyte activation in the presence of rIL-2

Additive to Thymocyte Suspension	[^{3}H]Thymidine Incorporation (cpm) per 10^6 Thymocytes		
	Expt. 1	Expt. 2	Expt. 3
Media	2,000	800	800
SRBC	4,900	700	400
SRBC + 35.1	9,600	4,500	700
SRBC + 9-1	8,200	5,000	600
SRBC + CD2.1	4,400	800	900
rIL-2	5,100	5,900	2,000
SRBC + rIL-2	4,500	3,800	2,900
SRBC + 35.1 + rIL-2	7,200	3,100	3,200
SRBC + 9-1 + rIL-2	78,500	26,100	43,400
SRBC + CD2.1 + rIL-2	46,800	24,100	15,000

[a] Thymocytes were prepared and cultures initiated as described in *Material and Methods*. SRBC were added at a concentration of 3×10^6 SRBC per 1×10^5 thymocytes. rIL-2 was added at 1 U/ml. Data shown are [^{3}H]thymidine incorporation after 4 days in culture.

no thymocyte activation (Table IV). In contrast to purified LFA-3 Ag, the combination of SRBC with anti-CD2 and anti-CD28 antibodies failed to induce thymocyte activation (not shown).

DISCUSSION

In this report, we demonstrate directly that purified LFA-3 Ag can activate human thymocytes via the CD2 pathway. However, LFA-3 Ag added alone to thymocyte suspensions did not induce thymocyte activation. Rather, induction of thymocyte activation occurred when purified LFA-3 Ag was added in combination with certain anti-CD2 antibodies (CD2.1 or 9-1) and exogenous rIL-2 (Table I) or when added in combination with anti-CD2 and anti-CD28 antibodies (Table II). In addition, intact SRBC expressing the sheep homolog of the human LFA-3 molecule, T11TS, also activated thymocytes in combination with CD2 antibodies CD2.1 or 9-1 and rIL-2 (Table IV).

Purified LFA-3 Ag has been shown to bind to human T cells via CD2 molecules (18) and to aggregate Jurkat T cells (18) and thymocytes (L.W. Vollger, B.F. Haynes, and K.H. Singer, manuscript in preparation). Furthermore, recent mutational analysis of the CD2 gene has confirmed binding of CD2 to cell surface LFA-3 molecules and identified the binding sites of multiple CD2 antibodies (31). Three general groups of CD2 antibodies have been defined (4, 32). mAb to the $T11_1$ epitope bind to all T cells and block T cell-SRBC rosetting, antibodies to the $T11_2$ epitope bind all T cells but do not block T cell-SRBC rosetting, and antibodies to the $T11_3$ epitope bind only to activated T cells (4). Although this analysis of CD2 epitopes provides a useful model, subsequent studies have revealed heterogeneity within each CD2 epitope group (5, 31). Meuer et al. (4) described T cell activation induced by simultaneous binding of antibodies to the $T11_2$ and $T11_3$ epitopes of the CD2 molecule, although later studies have demonstrated T cell activation by simultaneous binding of antibodies to $T11_1$ and $T11_2$ as well as to $T11_1$ and $T11_3$ epitopes (4–6).

In the present study, thymocyte activation by LFA-3 Ag required the presence of both antibody CD2.1 and exogenous rIL-2. Antibody CD2.1 has previously been reported to be mitogenic in combination with $T11_3$ anti-CD2 antibodies, does not block E rosettes, and has been grouped in the $T11_2$ anti-CD2 antibody subgroup (5, 32). Additionally, antibody CD2.1 was mitogenic in combi-

nation with certain $T11_1$, as well as $T11_3$ antibodies (5, 32). Since the anti-CD2 $T11_2$ antibody CD2.1 + LFA-3 Ag activated thymocytes, LFA-3 Ag in our study most likely is acting as a $T11_1$ CD2 ligand. The observation that LFA-3 Ag plus the $T11_3$ antibody, 9-1, also activated thymocytes in the presence of IL-2 (Table I) is also consistent with LFA-3 Ag serving as $T11_1$ ligand. The smaller proliferative response and, in certain experiments, absence of a proliferative response observed with 9-1 antibody and LFA-3 Ag stimulation (Tables I and II) is likely due to binding of 9-1 to a different epitope of CD2. However, we cannot rule out different binding affinity related to Ig subclass (9-1 is IgG3, whereas CD2.1 is IgG1). Finally, studies demonstrating that CD2 $T11_1$ antibodies inhibit the binding of LFA-3$^+$ thymic epithelial cells to CD2$^+$ thymocytes (18) further supports the notion that LFA-3 Ag binds via the $T11_1$ epitope of CD2.

Involvement of LFA-3 binding to T cell CD2 in peripheral T cell activation was initially demonstrated by Hunig (33) by using intact SRBC and a $T11_3$ anti-CD2 mAb. Subsequent experiments on human peripheral T cells with purified LFA-3 or T11TS in combination of $T11_2$ + $T11_3$ anti-CD2 antibodies (34) and on murine T cell hybrids transfected with CD2 DNA (35) have also suggested that LFA-3 Ag could induce peripheral T cell activation. Purified LFA-3 Ag induced proliferation of resting peripheral blood T cells in combination with submitogenic $T11_2$ + $T11_3$ antibodies (34) or antibody CD2.1.[4] In the latter case it was also shown that LFA-3 and antibody CD2.1 stimulated Ca^{2+} mobilization in resting PBL and in the Jurkat T leukemic cell line.

Anti-CD28 antibodies have been reported to augment T cell proliferation in response to a variety of stimuli (28–30, 36, 37). Yang et al. (38) have reported that anti-CD28 antibodies augment mature thymocyte proliferation to the anti-CD2 antibody combination, 9.6 and 9-1, via induction of CD1$^-$ thymocyte Il-2 production. In our studies, an anti-CD28 antibody (Kolt2) alone had no effect upon thymocyte proliferation, but markedly augmented thymocyte proliferation induced by LFA-3 Ag and CD2.1 antibody. In the present study, we directly demonstrated that LFA-3 Ag-dependent thymocyte activation in the presence of anti-CD28 antibody reflected proliferation of CD1$^-$ thymocytes (Fig. 4*B*). Thus, anti-CD28 antibodies likely augmented LFA-3 Ag-induced thymocyte proliferation via the CD2 pathway by increasing thymocyte IL-2 production. Evidence supporting this hypothesis was the demonstration that rIL-2 would support thymocyte activation by LFA-3 Ag and antibody CD2.1 in the absence of anti-CD28 antibody (Table I, Fig. 4*A*), and by the inhibition of LFA-3 Ag activation in the presence of anti-CD28 by anti-TAC and anti-IL-2 antibodies (Fig. 3).

The combination of LFA-3 Ag, CD2.1, and rIL-2 induced proliferation of both CD1$^+$ and CD1$^-$ thymocytes (Fig. 4*A*). Even highly purified CD1$^+$ thymocytes (95% CD1$^+$) proliferated in response to the combination of LFA-3 Ag, CD2.1, and rIL-2. Havran et al. (39) and Boyer and Rothenberg (40) have shown that CD4$^+$, CD8$^+$ thymocytes exhibited calcium mobilization and protein kinase C activation in response to CD3 antibody or phorbol esters but could not secrete IL-2, express IL-2R, or proliferate. This inability to secrete IL-2 or express IL-2R is consistent with our observation that CD1$^+$ thymocytes did not proliferate in response to the combination of LFA-3 Ag, CD2.1 antibody, and CD28 antibody. The proliferative potential of CD1$^+$ thymocytes in response to CD2 and rIL-2 stimulation is currently under active investigation.

Blocking studies by using anti-CD3 antibodies are consistent with the hypothesis that thymocyte activation via the CD2 pathway is regulated by the CD3-Ti complex.

Our studies of thymocyte activation with SRBC also demonstrated that thymocyte CD2 interactions with the SRBC homolog of human LFA-3, T11TS, can induce thymocyte activation. Similar to purified human E LFA-3 Ag, SRBC T11TS in conjunction with antibody CD2.1 delivered activation signals leading to thymocyte proliferation only when exogenous IL-2 was provided (Table IV). SRBC in combination with antibody CD2.1 and Kolt2 did not induce thymocyte activation, whereas purified LFA-3 Ag in this setting could induce thymocyte activation. A potential explanation for this difference is that purified LFA-3 Ag is multivalent and can more efficiently cross-link CD2 molecules necessary for thymocyte activation and stimulate thymocyte IL-2 production, whereas T11TS on SRBC is constrained by the red cell membrane and cannot cross-link CD2 molecules to stimulate sufficient thymocyte IL-2 production in the absence of exogenous IL-2.

Anti-CD28 + anti-CD2-dependent thymocyte triggering has previously been shown not to involve the most immature thymocyte subset, i.e, the CD4$^-$, CD8$^-$ (double negative) pool of thymocytes, presumably because human double-negative thymocytes are CD28$^-$ (38) (our unpublished observations). However, in a separate study, we have recently shown that purified double-negative thymocytes can indeed be triggered via the CD2 pathway in the presence of mitogenic anti-CD2 antibodies and IL-1 or IL-2 (41). Moreover, Blue et al. (42) have suggested that in the presence of exogenous monocytes and rIL-2, subsets of immature inner cortical thymocytes could also be activated via the CD2 pathway. Whether purified LFA-3 Ag can directly activate subsets of double-negative thymocytes is currently under study.

Our demonstration that purified LFA-3 Ag induces CD1$^-$ thymocyte activation via the CD2 pathway suggests that the LFA-3 molecule can serve as a functional ligand for thymocyte CD2 molecules. Ultimate understanding of intrathymic CD2 triggering and its relevance to T cell development will depend on defining natural ligands of the CD2 molecule other than LFA-3 that synergize with LFA-3 in activating thymocytes (43).

Acknowledgments. The authors thank Drs. John Hansen, A. J. McMichael, Soo Young Yang, Bo DuPont, Thomas Waldmann, Kendall Smith, and Ellis Reinherz for gifts of monoclonal reagents, Cetus Corporation for the gift of rIL-2, and David Leslie for assistance with flow cytometry. The expert secretarial assistance of Kim McClammy and Joyce Lowery is gratefully acknowledged.

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