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Requirements for In Vitro Growth of Human Thymocytes

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Since it is difficult to study human thymocyte maturation in vitro, we have developed an in vitro thymocyte culture system which has allowed us to select the optimal growth conditions for thymocyte subpopulations. Three thymocyte subpopulations (CD3−CD1−, CD1+CD3−, and CD3+CD1−) were isolated by a single step percoll density gradient centrifugation and indirect panning procedure using anti−CD1 and anti−CD3 monoclonal antibodies, and their purity was checked by flow cytometry. The combination of concanavalin A (Con A), tetradecanoylphorbol acetate (TPA), and IL−2 was shown to be the most reliable stimulus for the proliferation of CD3−CD1− thymocytes for up to 15 days in a culture system in vitro. Flow cytometric analysis for the phenotypic change of CD3−CD1− thymocytes revealed a steady increase of CD3 antigen after a 3−day cultivation, whereas there was no change in CD1 antigen intensity. A combination of Con A and IL−2 was both sufficient and necessary to induce growth of CD3+CD1− thymocytes. The major population of immature cortical thymocytes (CD3−CD1+ or CD3+CD1+), which are considered to be the most unresponsive dead−end cells, could not be maintained or stimulated with any combination used in this experiment, even in the presence of thymic accessory cells.

Key Words : *Human thymocyte, In vitro culure, IL−2 receptor expression, Con A receptor*

INTRODUCTION

During development, the uncommitted T cell precursors acquire specific antigen receptors which show MHC restriction and develop their functional program (Park et al., 1988; Reinherz et al.,1980; Royo et al.,1987; Sandberg et al.,1983; Scollay et al.,1985). Thymocytes undergo a complex series of genotypic and phenotypic changes during maturation in the thymus involving qualitative and quantitative change in the expression of cell surface differentiation antigens.

In human, discrete stages of intrathymic ontogeny have been defined on the basis of the monoclonal antibody probes directed at unique T-lineage specific surface antigens (Reinherz et al.,1980; Born et al., 1987; Janossy et al.,1981; Lobach et al.,1985; Toribio et al.,1986). Thus, the earliest identifiable T cell (Stage I) expresses CD7 but not CD1 or CD3 antigens (Piantelli et al.,1986). With further maturation, the CD1 molecule is acquired with coexpression of CD4 and CD8 molecules (Stage II) (Scollay et al., 1985). Stage I and Stage II thymocytes are mainly located in the thymic cortex representing about 2−10% and 70% of the total cell population, respectively. The most mature

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intrathymic pool (Stage III), mainly found in the medulla, lacks CD1 marker and expresses CD3 membrane molecules with either CD4 or CD8 molecules. Acquisition of the CD3 clonotypic receptor complex takes place in the last stage of ontogeny, although T cell receptor genes are rearranged in the earlier stages.

The functional development of thymocytes, such as MHC restriction and tolerance induction, is influenced by thymic epithelial and stromal elements (Royo et al., 1987). The details of intrathymic T cell ontogeny are, however, not well understood and still require experimental studies in vitro (Born et al., 1987;Janossy, 1980; d'Angeac et al., 1986; Palacios et al., 1986). For this reason, in this report we describe the isolation of various thymic subsets with high purity using the panning procedure, along with an investigation of the basic requirements for the growth of 3 thymocyte subsets. The changes of cell surface antigens during the proliferation process as well as the kinetics of the activation and proliferation of the thymocyte subsets are also described with specific reference to CD3−CD1− subsets.

MATERIALS AND METHODS

Tissue Culture Medium: Medium RPMI−1640 (GIBCO, Grand Island, NY) containing 2 mM L−glutamate and 20% heat−inactivated fetal calf serum (FCS, Hyclone, Sterile Systems Inc., Logan UT) was used in all the experiments, occasionally supplemented with 10^5 IU/L penicillin G potassium and 0.1g/L streptomycin sulfate.

Reagents: The calcium ionophore (Ca ionophore) A23187 (Sigma) was stored as a 1 mg/ml stock in dimethylsulfoxide at −20 ℃. The tumor promoter tetradecanoyl phorbol acetate (TPA) (Sigma) was stored as a 10 μg/ml stock in 95% ethanol. Concanavalin A (Con A) and fluorescein isothio-cyanate (FITC)−conjugated Con A were purchased from Pharmacia (Uppsala, Sweden). In all the experiments, human recombinant interleukin 2 (IL−2; Genzyme, Suffolk, England) was used.

Fractionation and Phenotyping of Thymocytes: Thymocyte suspensions were prepared, as previously reported (Goust et al.,1981), from fragments of thymuses that had been removed during corrective cardiac surgery of patients one to 12 months old. Viable thymocytes were isolated by Ficoll-Hypaque density centrifugation. For the enrichment of the thymocytes, a cell suspension was layered on to 53% percoll RPMI mixture and then centrifuged (2200 rpm) for 20 min at 4℃. Thymocytes were isolated from the interface of the gradient and washed twice in cold media. Thymocytes were separated into 3 subsets on the basis of their reactivity with anti−CD3 and anti−CD1 monoclonal antibodies using a modified panning procedure (Wysocki et al.,1978). Briefly, 10 cm Petri dishes were coated with 4 ml of an 83 μg/ml solution of goat anti-mouse IgGAM for 2 hours at room temperature and then washed 5 times with cold PBS. The Petri dishes were then stored at 48 overnight in PBS. Thymocytes were incubated with OKT3(CD3) and/or OKT6(CD1) mono-clonal antibodies (Ortho Diagnostics, Raritan, NJ) for 40 min at 4℃, and $20x10^6$ cells were incubated in each Petri dish at 4℃ for 90 min. The unbound thymocytes were harvested by Pasteur pipettes after gentle swirling of the dishes. CD3−CD1− thymocytes reactive with peanut agglutinin (PNA) were also selected by panning procedure. After recovery, the cells were stained with OKT3 and/or OKT6 monoclonal antibodies and FITC−coupled goat anti−mouse Ig. The purity of the relevant cells was >95%.

Proliferation Assay: The fractionated thymocytes were resuspended in medium, and triplicate cultures of 10^4 cells/0.2 ml were established in microtiter U−bottomed wells (Microtest II plate, Falcon 3040). The following agents were added either alone or in several combinations: Con A (10 μg/ml), TPA (10 ng/ml), calcium ionophore (Ca ionophore) A23187 (300 ng/ml), and rIL−2 (100 U/ml). The cells were cultured for 3, 6, 9, 12, and 15 days. For the last 18 hours of the culture period, 0.5 μCi of (methyl−3H) thymidine (3H−TdR: specific activity 5 Ci/

mmol Radiochemical Center, Amersham, England) was added to each culture well. At the end of incubation, the cells were collected on a Titertek harvester (Flow Laboratories, Irvine, Scotland), and the radioactivity was measured. Routine assays of spontaneous proliferation were performed during each culture period and in the absence of reagents described above.

Quantitative Flow Cytometry: CD1 and CD3 staining was performed using fluorescein conjugated $F(ab')_2$ goat anti-mouse IgG second-step reagent (Kisielow et al.,1984), and the cells were analyzed with a dual laser FACS 440 flow cytometer (Becton Dickinson). Cells that stained above the second-step reagent background were considered positive.

Table 1. Proliferation of CD3-CD1-Thymocyte

	3H-Thymidine Incorporated (cpm)													
Culture	Exp 1		Exp 2		Exp 3		Exp 4		Exp 5		Exp 6		Exp 7	
Period(day)	3	6	3	6	3	6	3	6	3	6	3	6	3	6
Medium	254	465	414	298	93	408	93	59	66	92	89	115	95	290
Con-A (10 μg/ml)	321	451	816	1348	129	333								
IL-2 (100 U/ml)	870*	5399*	1662*	1640*	2403	8416	878	2596	7856	6593	431	4972	287	820
TPA (10 ng/ml)	847	943	612	720	165	390								
Ca Iono (300 ng/ml)	194	359	622	540	247	381								
Con-A+IL-2	735*	2891*	534*	1136*	2539	9589								
Con-A+Ca Iono	263	309	562	552	347	607								
Con-A+TPA	1102	1311	1384	870	360	728								
TPA+IL-2	4487*	15722*	3030*	3222*	2673	8666	1601	3590	8848	10499	1583	16080	1623	–
TPA+Ca Iono	741	623	686	566	346	545								
ConA+TPA+Ca Iono	926	993	1180	1490	402	781								
ConA+TPA+IL-2	3161*	13525*	2026*	4155*	3000	24263	1882	14020	14680	19244	2761	27088		
ConA+Ca Iono+IL-2	587*	2032*	1370*	1083*	2662	–								
TAP+Ca Iono+IL-2	3755*	10408*	3038*	3564*	3689	12282	641	6094	11811	10811	2460	16142		

Thymocytes were seperated by a modified panning procedure using OKT3(CD3) and/or OKT6(CD1) monoclonal antibodies. CD3-CD1-thymocytes were cultured with the designated agents and after 3 or 6 days of culture period. 3H thymidine uptake was measured.

*IL-2 was used at the concentration of 20 u/ml(*) or 100 u/ml. Tetradecanoylphorbol ester (TPA), Concanavalin A (Con-A), and calcium ionophore A23187 (calono) were used at the given concentrations either alone of in several combinations.

RESULTS

The inducer agents acting either on the cell membrane (e.g., Con A) or mimicking the action of signal transducers (e.g., Ca ionorhore, TPA) and well characterized growth factors (IL−2) were tested alone or in combination for their capacity to induce the growth of thymocytes in 3 different developing stages. The proliferative responses were compared after culture periods of 3 days and 6 days.

CD3−CD1− Thymocytes : The combination of TPA, Con A, and IL−2 consistently induced the best growth responses in the culture of CD3−CD1− thymocytes. Also observed were considerable growth responses with combinations of Con A + IL−2 and TPA + Ca ionophore + IL−2 (Table 1). The IL−2 alone and in combination with the other inducing agents also induced a significant proliferation of the CD3−CD1 − prethymocytes.

Somewhat higher proliferative responses were induced in the 6−day culture period than in the 3−day culture period, but neither any single agent nor any other combination of agents induced significant growth responses.

In the long−term cultures of the CD3−CD1− thymocytes up to 15−day culture period with the inducing agents or the growth factor alone or in various combinations, the combination of Con A, TPA, and IL−2 induced the best growth response (Table 2).

CD3−CD1+ Thymocytes : It is well −known that most of the immature thymocytes with a phenotype of CD1 fail to differentiate into functional T lymphocytes and ultimately die in the thymus. The result of this assay with the CD3−CD1+ thymocyte was comparable to those of previous reports by many authors.

Any kind of combination could not induce a proliferative response of the CD3−CD1+ thymocyte, even if there was an increased thymidine uptake of a mild degree. This mild proliferative response was thought to be due to the possible presence of either con-

Table 2. Long−term Culture of CD3−CD1− Thymocyte

	3H−Thymidine incorporated (cpm)							
	Exp 1					Exp 2		
Culture Period(day)	3	6	9	12	15	3	6	9
Media	378	1280	674	838	333	367	506	433
IL−2	3994	9768	−	−	−	2401	3966	3761
Con−A+IL−2	4065	11983	−	−	−	3015	5116	4990
TPA+IL−2	4862	18540	22781	23996	29628	2696	4071	5046
TPA+Ca Iono+IL−2	4819	14741	18750	37144	50289	2633	3644	3012
Con−A+TPA+IL−2	5709	22371	31301	42141	82803	3142	6285	30185

CD3−CD1−thymocytes were measured for 3H thymidine uptake after the given culture period. The concentration of stimulating agents was the same with table 1 (IL−2; 100u/ml).

Table 3. Proliferation of CD3−CD1+ Thymocyte

Stimulus	3H−Thymidine Incorporated (cpm)				
	Exp 1	Exp 2		Exp 3	
Culture Period(day)	3	3	6	3	6
Media	36	174	91	209	288
Con−A (10 μg/ml)	152	236	133	357	344
IL−2 (20 U/ml)	83	69	111	331	343
TPA (10 ng/ml)	137	166	113	314	262
Ca Iono (300 ng/ml)	29	105	148	348	229
Con−A+IL−2	156	479	390	283	264
Con−A+Ca Iono	54	126	80	327	214
Con−A+TPA	345	159	92	486	289
Con−A+TPA+Ca Iono	325	219	83	211	385
Con−A+TPA+IL−2	398	337	349	407	535
Con−A+Ca Iono+IL−2	232	482	351	284	284
TPA+IL−2	92	78	77	423	630
TPA+Ca Iono	44	150	165	259	295
TPA+Ca Iono+IL−2	787	180	197	418	563
Ca Iono+IL−2	72	104	119	165	290

CD3−CD1+ thymocytes were measured for 3H thymidine uptake after 3 or 6 days of culture period with the designated stimulating agents.

taminating CD3−CD1− thymocytes or CD3+ mature thymocytes (Table 3).

CD3+CD1− Thymocyte : Con A plus IL−2 was a necessary and sufficient requirement for the growth response of the CD3+ CD1− thymocytes (Table 4). Since Con A can bind to both the CD3 complex and the T−cell receptor on the T−cell surface, Con A is a rather more effective proliferation−inducing agent for the mature thymocytes than for the immature thymocytes. In conclusion, the combination of Con A plus IL−2 was the sufficient growth requirement for mature thymocytes.

Proliferation of the CD3−CD1−PNA+ or CD3−CD1−PNA− Thymocytes : The 2 subpopulations of the CD3−CD1− thymocytes are thought to be of different lineage. To acertain which subpopulation is responsible for the growth response of the CD3−CD1− thymocytes, the prethymocyte population was divided into 2 subpopulations according to the presence or absence of a PNA receptor.

Each prethymocyte subpopulation was assayed for growth response to the combina-

Table 4. Proliferation of CD3+CD1− Thymocyte

Stimulus	3H−Thymidine Incorporated (cpm)								
	Exp 1	Exp 2	Exp 3	Exp 4		Exp 5		Exp 6	Exp 7
Culture Period(day)	3D	3D	3D	3D	6D	3D	6D	6D	6D
Media	311	382	207	223	327	59	68	481	337
Con−A (10 μg/ml)	−	−	−	2025	266	−	−	860	598
IL−2 (20 n/ml)	230	2142	614	233	365	63	83	3307	395
TPA (10 ng/ml)	186	−	−	597	53	−	−	445	289
Ca Iono (300 ng/ml)	98	−	−	55	155	−	−	172	290
Con−A+IL−2	3652	58315	4915	10207	28697	2293	6725	22537	1540
Con−A+Ca Iono	1522	−	−	3133	151	−	−	203	301
Con−A+TPA	3136	−	−	3036	776	−	−	537	391
Con−A+TPA+Ca Iono	2094	−	−	1948	1385	−	−	568	675
Con−A+TPA+IL−2	1828	32705	2582	3116	1134	1421	4662	493	613
Con−A+Ca Iono+IL−2	2752	−	−	9125	11836	−	−	19709	715
TPA+IL−2	137	2962	353	249	274	71	151	3406	817
TPA+Ca Iono	140	−	−	54	48	−	−	330	184
TPA+Ca Iono+IL−2	174	3629	394	2994	2474	171	116	4299	720
Ca Iono+IL−2	321	−	−	139	121	−	−	1908	270

CD3+CD1− thymocytes were measured for 3H thymidine uptake after 3 or 6 days of culture period with the designated stimulating agents.

tions of Con A + TPA + IL−2 and TPA + Ca ionophore + IL−2. Both subpopulations showed identical proliferative responses to the 2 combinations of agents (Table 5).

Phenotypic Change of the Cultured Thymocytes of Different Differential Stages.

A change of the surface thymocyte differentiation antigen was observed. The CD3+ cells slightly increased in the CD3−CD1− subpopulation after 6−day culture (Fig. 1). This may be interpreted as cellular differentiation during the culture period, but the possibility of contamination of the CD3+ cells was not excluded. Any antigenic change was not observed in the CD3+CD1− thymocyte subpopulation (Fig. 2). This suggested that the cell preparation of the CD3+ CD1− thymocyte was of high purity.

DISCUSSION

The thymocytes of the earliest stage (prethymocytes) expressed only the CD7 and CD2 molecule (Piantelli et al., 1986). This population is represented as CD3−CD1− in this paper and accounts for 5−10% of the total thymocytes. The results of this assay were different from the studies previously

Table 5. Proliferation of Two Groups of CD3−CD1− Thymocytes with Low and High PNA Receptors

	3H−Thymidine Incorporated							
	EXP. 1				EXP. 2			
PNA	+		−		+		−	
Culture Period (day)	3D	6D	3D	6D	3D	6D	3D	6D
Medium	431	381	800	596	116	94	131	143
IL−2	1340	1900	2455	2404	−	−	−	−
TPA+IL--2	3217	1824	3060	2662	−	−	−	−
TPA+Ca Iono+IL−2	2500	1700	2463	2809	4630	1331	6563	741
Con−A+TPA+IL−2	1764	2559	2602	3622	4756	38929	6347	23204

CD3−CD1− thymocytes were measured for 3H thymidine uptake after 3 or 6 days of culture period with the designated stimulating agents in two PNA receptor − positive or PNA receptor − negative subpopulation.

described, which had been done in the mouse system (Raulet,1985). They reported that the CD3−CD1− thymocytes did not express Con A receptors. However, this finding was not the case in the human system because the CD3−CD1− human thymocytes showed a high number of Con A receptor expressions by flowcytometric analysis in a reproducible way. It is our understanding that the differences in receptor expression between the CD3−CD1− thymocytes of humans and mice might explain the discrepancies in the results between these 2 reports. Taken together, the combination of IL−2 and TPA is the basic requirement for the in vitro growth of double negative thymocytes. Addition of Con A to this combination of IL−2 and TPA augmented and prolonged the growth response of this most immature thymocyte.

It has been reported that the IL−2 receptor is expressed in 50−70% of the CD3−CD1− thymocytes (Ceredig et al., 1985 and Raulet, 1985), suggesting the importance of IL−2 in the proliferation and differentiation of the immature thymocyte (Lowenthal et al., 1986; Lugo et al.,1985 and 1986). But the exogenous IL−2 alone could not induce the proliferation of the CD3−CD1− cells. The CD3−CD1− thymocytes showed 3−4 times less affinity for IL−2 than the mature thymocyte. In contrast, Zlotnik et al.(1987) have suggested that IL−4 is the proliferating factor for the activated mature thymocytes and fetal thymocytes, and that the proliferation of the CD3−CD1− thymocytes can be induced by a combination of IL−4 and TPA but not by IL−4 or TPA alone and was IL−2 independent.

Caplan and Rothenberg (1984) reported that the best requirement for IL−2 release of the thymocytes is the combination of

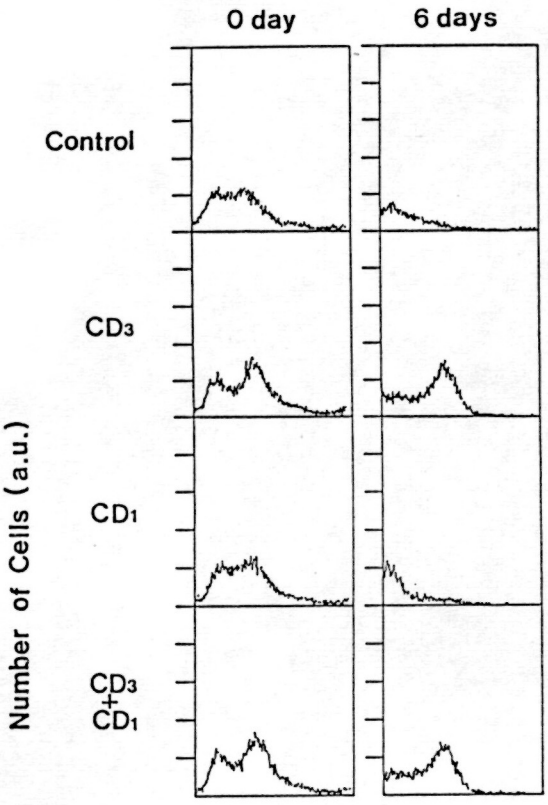

Fig. 1. Phenotypic properities of CD3-CD1- thymocytes selected by panning procedure, and 6-day cultured CD3-CD1- thymocytes. Cells (0 day thymocytes - 4 panels, left or 6-day cultured cells-4 panels, right) were harvested and stained by indirect immunofluorescence with mAb to CD3 and CD1, followed by goat anti-mouse FITC second step reagent and analysis by cytoflowmetry. Control cells were stained with the second step reagent alone. Each histogram shows the fluorescence intensity (horizontal scale, arbitrary units: a.u.) of 10,000 cells.

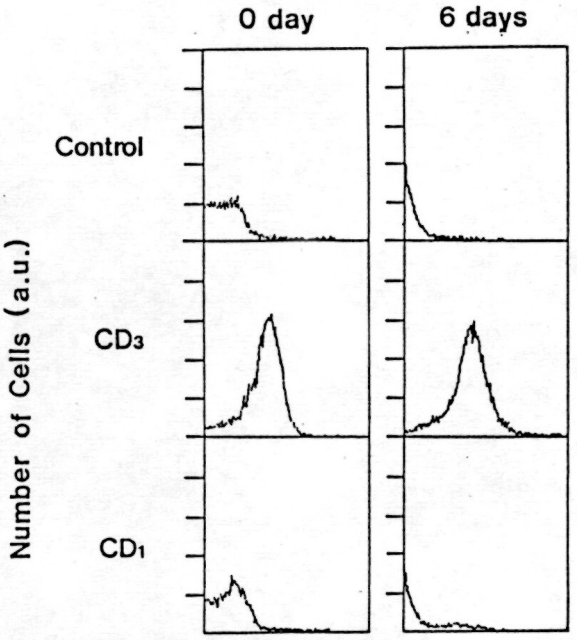

Fig. 2. Phenotypic properties of CD3+CD1− thymocytes selected by panning procedure, and 6-day culture CD3+CD1− thymocytes. Cells (0 day thymocytes − 3 panels, left or 6-day culture − 3 panels, right) were harvested and stained by indirect immunofluorescence with mAb to CD3 and CD1, followed by goat anti-mouse FITC second step reagent and analysis by cytoflowmetry. Control cells were stained with the second step reagent alone. Each histogram shows the fluorescence intensity (horizontal scale, arbitrary units : a.u.) of 10,000 cells.

Con A and TPA. Lugo et al. (1986) notes that the CD3−CD1− thymocytes respond only to TPA + Ca ionophore but not to Con A + TPA, and that the mature thymocytes respond to both combinations. In our study we have confirmed that CD3−CD1− thymocytes have not expressed the IL−2 receptor throughout the 3−day culture period using indirect immunofluorescent study by anti−Tac antibody when activated by the combination of Con A and TPA (data not shown). As an alternative way to detect the presence of putative IL−2 receptor−bearing cells, we studied the responsiveness of CD3−CD1− thymocytes toward IL−2 in vitro. As shown in Table 1, IL−2 induced some proliferation in the double negative thymocyte population, suggesting that CD3−CD1− thymocytes express IL−2 receptors in low but sufficient density for an IL−2 dependent proliferation to take place. Roosnek et al. (1986) have also demonstrated that extensive depletion of Tac−positive cells from peripheral T cell populations does not abolish the IL−2 responsiveness of the remaining T cells. Taken together these observations suggest that the Tac−negative cell population still contains a subset of IL−2 receptor−bearing T cells. It is likely that these cells express the other p75 chain of the IL−2 receptor. The relative paucity of

the Tac antigen (p55) has also been reported by Plum and de Smedt (1987).

In the present study, we could not induce a growth response of the CD3−CD1+ immature thymocytes by any combination of inducing agents and growth factors. These results are comparable to the previous results and suggest that CD3−CD1+ cells are end cells which remain unresponsive to all combinations of stimuli currently known to have some relationship to thymocyte proliferation (Piantelli et al., 1986). In the third stage of thymocyte differentiation, the mature thymocytes express CD3 antigen but not CD1 antigen. This population is phenotypically similar to peripheral T lymphocytes and does not have the affinity for PNA. The combination of IL−2 and Con A induced the best growth response of this CD3 +CD1 mature thymocyte population suggesting that Con A is a stronger inducer of mature thymocyte than the immature thymocyte population. It is known that Con A bind to both the CD3 complex and the T−cell receptor (Palacios and Von Boehmer, 1986).

In summary, any combination of stimuli containing high concentrations of IL−2 (more than 100 u/ml) in the presence of accessory cells presents a basic requirement for the growth of CD3−CD1− thymocytes in the short−term culture period, and the combination of Con A, TPA, and IL−2 is the most suitable for the long−term growth of double negative thymocytes. At the same time the combination of Con A and IL−2 is both sufficient and necessary to induce growth of CD3+CD1− thymocytes, while the major population of immature thymocytes (CD3−CD1+ or CD3+CD1+) cannot be induced to proliferate with a combination of any of these stimuli.

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