

Dendritic Cell Development in Culture from Thymic Precursor Cells in the Absence of Granulocyte/Macrophage Colony-stimulating Factor

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Summary

The earliest lymphoid precursor population in the adult mouse thymus had previously been shown to produce not only T cells, but also dendritic cell (DC) progeny on transfer to irradiated recipients. In this study, culture of these isolated thymic precursors with a mixture of cytokines induced them to proliferate and to differentiate to DC, but not to T lineage cells. At least 70% of the individual precursors had the capacity to form DC. The resultant DC were as effective as normal thymic DC in the functional test of T cell stimulation in mixed leukocyte cultures. The cultured DC also expressed high levels of class I and class II major histocompatibility complex, together with CD11c, DEC-205, CD80, and CD86, markers characteristic of mature DC in general. However, they did not express CD8 α or BP-1, markers characteristic of normal thymic DC. The optimized mixture of five to seven cytokines required for DC development from these thymic precursors did not include granulocyte/macrophage colony stimulating factor (GM-CSF), usually required for DC development in culture. The addition of anti-GM-CSF antibody or the use of precursors from GM-CSF-deficient mice did not prevent DC development. Addition of GM-CSF was without effect on DC yield when interleukin (IL) 3 and IL-7 were present, although some stimulation by GM-CSF was noted in their absence. In contrast, DC development was enhanced by addition of the Flt3/Flk2 ligand, in line with the effects of the administration of this cytokine *in vivo*. The results indicate that the development of a particular lineage of DC, probably those of lymphoid precursor origin, may be independent of the myeloid hormone GM-CSF.

Dendritic cells (DC)¹ (1) are generally considered as relatives of monocytes and macrophages and to be of myeloid origin. Strong support for this view comes from many studies on the outgrowth of DC in culture, induced principally by GM-CSF, usually in association with other cytokines including TNF- α (2–11; for review see reference 12). The DC appear to derive from a progenitor also capable of forming granulocytes and macrophages (13), although more recently a committed DC progenitor, possibly a downstream precursor, has been identified (14). The myeloid nature of DC is emphasized by the direct development of a form of DC from blood monocytes (9, 15–17; for review see reference 12). All these lines of evidence for a myeloid origin of DC derive from culture studies using GM-CSF.

In contrast to these studies, we have used adoptive transfer of highly purified precursor cells isolated from the mouse thymus to demonstrate that certain types of DC are related to the lymphoid lineage. The earliest T precursor population isolated from the adult mouse thymus, the “low CD4 precursor,” was unable to form detectable erythroid or myeloid cells, yet had the potential to form T cells, B cells, NK cells, and DC (18–23; for review see reference 24). A progenitor cell with similar developmental potential has since been isolated from human bone marrow (25). T cells and DC bearing CD8 α , a characteristic of murine thymic DC (26), developed in parallel when this low CD4 precursor was transferred directly into a recipient thymus (21). We have recently found that a downstream thymic precursor (CD4⁻⁸-44⁺²⁵+c-kit⁺), now no longer able to form B cells or NK cells, still retains full capacity to form DC as well as T cells, suggesting a strong relationship between the two lineages (27).

¹Abbreviations used in this paper: CD40L, CD40 ligand; DC, dendritic cells; Flt3L, Flt3/Flk2 ligand; SCF, stem cell factor.

These thymic precursors also formed CD8 α ⁺ DC in the spleen after intravenous transfer, suggesting that the CD8 α ⁺ DC normally found in peripheral lymphoid tissues might also be of lymphoid origin. These CD8⁺ splenic DC appear to have a regulatory role in T cell responses (28).

Our initial attempts to grow the thymic low CD4 precursors in culture under the influence of multiple combinations of up to three cytokines were unsuccessful. Some growth and development was obtained using an underlay of a thymic epithelial cell line; under these conditions, a limited production of DC was evident (29). However, more extensive growth, with development into DC rather than T cell progeny, was evident once a more complex cocktail of cytokines was used. It was notable that GM-CSF was not required for this DC development in culture.

Materials and Methods

Mice. The mice used for isolation of thymic low CD4 precursors, or for isolation of thymic DC, were usually 5–7 wk-old C57BL/6J Wehi females, bred under specific pathogen-free conditions at The Walter and Eliza Hall Institute animal facility. The GM-CSF-null mice, produced at the Ludwig Institute (30), were originally on a C57BL/6 \times 129 background but had been backcrossed for five generations onto C57BL/6J mice; 5–9-wk-old males and females were used. The source of the CD4⁺ LN T cells for mixed leukocyte reactions was 5–6-wk-old female CBA/J mice bred under specific pathogen-free conditions at The Walter and Eliza Hall Institute.

Isolation of Normal DC from the Thymus. The procedure was modified from that given in detail elsewhere (26, 28, 31). Briefly, pooled thymuses from 10 mice were cut into fragments, and the entire tissue was digested for 25 min at 22°C with collagenase-DNase. The digest was incubated a further 5 min with EDTA to break up DC-T cell complexes. Light density cells were then isolated from the digest by centrifugation at 4°C in a 1.077 g/cm³ density medium isoosmotic with mouse serum. The light-density cells were then coated with a cocktail of mAb reactive with CD3, CD4, Thy 1, CD25, B cell antigen B220, erythrocyte antigen TER119, granulocyte antigen Gr-1, macrophage antigen F480, FcR2 and CD11b, and then the coated cells were removed using anti-Ig-coated magnetic beads. Finally the DC in the enriched preparation were stained and sorted as cells excluding propidium iodide, with the high forward and side scatter of DC, and expressing relatively high levels of CD11c (28).

Preparation of Low CD4 Thymic Precursor Cells. An improved procedure was used, modified from our original method (18) and given in detail elsewhere (32). Briefly, the lightest 30% of thymocytes were selected by a density cut procedure, and then all adherent cells were removed by culture for 1 h in Petri dishes. Cells bearing CD3, CD8, CD25, B220, class II MHC, Gr-1, Mac-1, and the erythrocyte antigen TER 119 were all removed by first coating with mAb, then removing coated cells with two rounds of anti-Ig-coated magnetic beads. Finally, the 1% remaining thymocytes were stained with anti-Thy 1 (PE-conjugated 30.H12) and anti-c-kit (FITC-conjugated ACK-2). The low CD4 precursors were sorted as cells low but positive for Thy 1 and moderate to strongly positive for c-kit. The preparation was >97% pure on reanalysis by these markers and appeared homogeneous by 14 other markers tested in previous experiments (18–21, 24, 32). In some experiments, the identical procedure was applied

to bone marrow cells, but the homogeneity of this preparation was not assessed.

Culture Conditions. The culture medium was based on RPMI 1640, modified to be isoosmotic with mouse serum (308 mosM), with additional Hepes buffering at pH 7.2 and supplemented with 10% FCS, 10⁻⁴ M 2-ME, sodium pyruvate, and antibiotics. The required cytokines were then added to the medium, and the precursor cells were dispersed in the mix. Cultures were from 1 to 7 d at 37.5°C in a humidified 10% CO₂-in-air incubator. For most studies the culture volume was 0.01 ml, and culture of 1–3,000 cells was in the wells of Terasaki trays (Nunc, Naperville, IL). When a larger cell yield was required for cell counts or for surface phenotype analysis, 20,000 precursors were cultured in 0.1 ml medium in flat-bottomed 96-well culture trays (Disposable Products Pty. Ltd., Adelaide, Australia).

Cytokines and Cytokine-related Antibodies. The following concentrations of recombinant cytokines tested were used for culture of the low CD4 precursors. IL-1 β (human), 200 U or 0.2 ng/ml; TNF- α (murine), 1 ng/ml; IL-3 (murine), 200 U or 400 ng/ml; IL-4 (murine), 200 U or 20 ng/ml; IL-7 (human), 200 U or 10 ng/ml; GM-CSF (murine), 200 “Immunex units” or 200 ng/ml. GM-CSF was found by titration at the end of the experimental series to be 3,200 standard units or the equivalent of 16 ng/ml of a standard Hall Institute preparation. These cytokines were all provided by Immunex Corp. Flt3/Flk2 ligand (Flt3L), 100 ng/ml, and antibody against GM-CSF, 2 μ g/ml, was provided by Dr. N. Nicola (The Walter and Eliza Hall Institute). CD40 ligand (CD40L) and mAb against CD40, FGK45.5, 1 μ g/ml, was provided by Dr. A. Rolink (Basel Institute for Immunology, Basel, Switzerland).

Cluster Counts, Cell Counts, and Visualization of Dendritic Morphology. The incidence of DC clusters was counted directly on the Terasaki tray cultures, using inverted phase-contrast microscopy; a group of >20 cells was considered a cluster. To recover and count cells after culture, one tenth volume of 0.1 M EDTA, pH 7.2, was first added to the warm cultures, and then the cultures were mixed by repeated passage through a pipette tip in order to break up the DC clusters into a single cell suspension; cell counts were then carried out in a hemocytometer using phase-contrast microscopy. To assess dendritic morphology, a cell suspension was prepared from pooled cultures using EDTA to aid dissociation, as above. The cells were then washed by centrifugation through a layer of FCS and resuspended in a small volume of culture medium. The suspension was placed in slide chambers, prepared by fastening square coverslips onto microscope slides by double-sided adhesive tape at two opposite edges. After filling the chambers, the remaining edges were sealed with nail polish. The slides were then incubated at 37°C for 1–2 h and then examined under phase-contrast microscopy. To monitor the fate of individual precursor cells, Terasaki tray cultures containing only a single precursor were selected after 2 h of incubation of cultures set up using 1 cell/0.01 ml medium, and then the culture was inspected every 24 h using inverted phase-contrast microscopy.

Immunofluorescent Staining and Flow Cytometry. The procedures used for staining the cultured DC were similar to those used previously for DC extracted from tissues (26). Two- or three-fluorescent-color staining was used, propidium iodide was used to exclude dead cells, and the samples were analyzed using a FACStar Plus[®] (Becton Dickinson & Co., San Jose, CA). The mAbs and the fluorochromes used were as follows: CD4, Cy5-conjugated H129.19.6.8; CD8 α , biotin-conjugated 53-6.7; CD8 β , biotin-conjugated 53-5.8; CD3, PE-conjugated KT3-1.1; class I MHC, biotin-conjugated M1/42; class II MHC, Texas red-conjugated

N22; CD11c, biotin-conjugated N418; DEC205, FITC-conjugated NLDC145; BP-1, biotin-conjugated 6C3; CD11b, FITC-conjugated M1/70; F480 macrophage antigen, biotin-conjugated F4/80; B220, biotin-conjugated RA3-6B2; CD80 (B7/1), biotin-conjugated 16-10A1; CD86(B7/2), biotin-conjugated GL-1; CD40, FITC-conjugated FGK45.5; CD44, FITC-conjugated IM7.81; Gr-1, FITC-conjugated RB6-8C5; and CD25, biotin-conjugated PC61. PE-conjugated streptavidin was used as the second stage for all biotin conjugates.

Mixed Leukocyte Cultures for Assessing CD4 T Cell Stimulatory Capacity. The cultures were set up and T cell proliferation was determined as described previously (28). Briefly, 100–2,000 DC of C57BL/6 origin, either harvested from the cultures or isolated from the thymus, were cultured with 20,000 purified CD4 T cells isolated from the LN of CBA mice. The culture medium was modified RPMI 1640, 0.1 ml being used in the wells of V-bottom 96-well culture trays. No exogenous cytokines were added. After 2–4 d at 37.5°C in a 10% CO₂-in-air incubator, the cultures were pulsed for 9 h with [³H]TdR. Cells in the cultures were harvested onto glass-fiber filters, and incorporated radioactivity was measured in a gas-flow scintillation counter.

Results

Culture of Low CD4 Precursors with One to Three Cytokines. In our initial studies, the low CD4 precursors were isolated from adult mouse thymus by depletion and sorting, and then they were cultured at 50–1,000 cells per well in Terasaki tray cultures with a range of recombinant cytokines. The cytokines were tested singly or in combinations of two or three. In no case of cytokines used alone or in combinations of up to three was any growth detected. This included the cytokines normally used to produce DC in culture, namely, GM-CSF or GM-CSF in combination with TNF- α and/or IL-4. It also included IL-2 and IL-6, not used in our subsequent studies.

However, almost all the cytokines, even when used singly, gave some improvement in low CD4 precursor survival. After 36 h of culture in medium alone, an average of only 10% of the precursors were viable. The cytokines, which, when used alone, increased survival to >30%, were IL-3, IL-6, stem cell factor (SCF), TNF- α , IL-4, and GM-CSF; IL-7 gave the best survival, 55%. No combination of cytokines gave a survival >45%. Some combinations of cytokines reduced survival to that of the medium alone, in particular, TNF- α with GM-CSF, TNF- α with SCF, TNF- α with IL-4, and IL-4 with IL-7. This indicates that low CD4 precursors expressed receptors for many of these cytokines but that the interactions between them were complex.

Growth and Differentiation of Low CD4 Precursors in Response to Multiple Cytokines. In contrast to this lack of proliferation in response to combinations of up to three cytokines, some growth and differentiation of the low CD4 precursors was obtained on thymic epithelial cell lines (29). This encouraged us to test a complex cocktail of seven cytokines, including some that might have been produced by thymic epithelial cells, namely, TNF- α , IL-1 β , IL-3, IL-4, IL-7, SCF, and GM-CSF. This cytokine cocktail was tested on

the purified precursors cultured alone, without the thymic epithelial cell underlay. It produced a definite growth of precursors, doubling the input cell number by day 3. A requirement for multiple cytokines to induce growth in the low CD4 precursors has also been reported by Moore and Zlotnik (23). However, in our cultures the end-product cells appeared to be DC. Cells with cytoplasmic extensions and DC morphology appeared by day 1. From day 2 to day 4 of culture, a high proportion of the cells formed large clusters of ~50 cells, resembling closely the DC clusters generated by culturing bone marrow or blood precursors with GM-CSF and other cytokines (2–9). The majority of cells in the cultures had the morphological appearance of DC by day 4.

The clusters appeared to form as a result of aggregation, rather than representing true colonies derived from single precursors. Nevertheless, over the range of 100–5,000 low CD4 precursor cell input, there was a dose-response relationship between cells cultured and clusters formed at day 4, with five to eight clusters being formed per 1,000 cells cultured (Fig. 1). Accordingly, scoring the number of large clusters formed in the Terasaki well cultures provided a rapid assay for proliferation and DC production. With relatively dense cultures (3,000 precursors per well) a statistically reliable estimate could be made with around five cultures per point. Such a cluster count was used as the initial readout for screening the contribution of different cytokines.

The Effect of Omitting Cytokines on DC Cluster Development. To determine which of the cytokines in the complex mix were essential, the effect of leaving out one or two individual cytokines from the initial mix was systematically assessed, using the incidence of DC clusters at day 4 as a readout (Fig. 2). Several cytokines (GM-CSF, IL-4, IL-7,

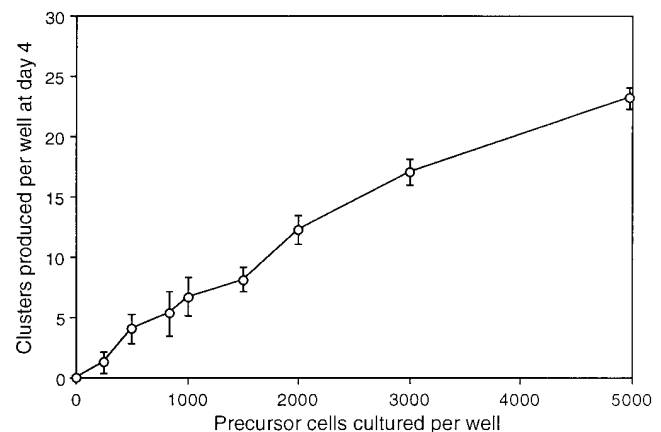


Figure 1. The relationship between the number of low CD4 precursors cultured and the number of DC clusters produced. Purified thymic low CD4 precursors were cultured for 4 d in 0.01 ml medium in Terasaki tray cultures. The number of clusters (>20 cells) per well was counted under phase-contrast microscopy. The initial complement of seven cytokines was used (TNF- α , IL-1 β , IL-3, IL-4, IL-7, SCF, and GM-CSF). The results are the means \pm SEM of pooled data from three experiments, each with five cultures per point.

% CLUSTERS FORMED

-IL1 β	66 \pm 7	Control					
-IL3	26 \pm 9	81 \pm 22	IL1 β +IL3+IL4+IL7+SCF+TNF α +GMCSF				
-IL4	66 \pm 9	98 \pm 7	96 \pm 18				
-IL7	11 \pm 4	42 \pm 2	72 \pm 2	101 \pm 12			
-SCF	26 \pm 12	77 \pm 8	77 \pm 12	46 \pm 10	97 \pm 13		
-TNF α	8 \pm 2	19 \pm 4	55 \pm 10	16 \pm 2	46 \pm 14	67 \pm 16	
-GMCSF	27 \pm 7	88 \pm 10	103 \pm 19	23 \pm 11	63 \pm 17	23 \pm 19	107 \pm 18
	-IL1 β	-IL3	-IL4	-IL7	-SCF	-TNF α	-GMCSF

Figure 2. The effect of omitting one or two cytokines from the initial mix of seven on the formation of DC clusters. All results are expressed as a percentage of the number of clusters formed with the complete control cytokine mix. The values given along the diagonal, outlined by the heavier boxes, represent single-cytokine omissions. Other boxes give results for two cytokine omissions. Thymic low CD4 precursor cells were cultured at 3,000 cells per well in Terasaki culture plates, and the number of clusters of DC were counted at day 4. The results are means \pm SEM of pooled data from three to five experiments with five cultures per condition per experiment.

SCF, and IL-3) could be omitted individually without much effect on cluster formation. However, their absence generally did have an effect if omitted along with another cytokine; one exception was the omission of GM-CSF and IL-4 together, where no drop was evident. The two cytokines whose omission, either alone or in combination with other cytokines, had the greatest effect were IL-1 β and TNF- α . When omitted together, cluster formation dropped to 8% of that seen with the complete mix. It was also notable that when GM-CSF was omitted, cluster formation became very dependent on IL-7. In contrast, the omission of both GM-CSF and IL-3, which share a common receptor chain (33) and might therefore have substituted for one another, caused only a small drop in cluster formation.

Accordingly, IL-1 β and TNF- α were considered essential components of the mix, whereas GM-CSF and possibly IL-4 appeared dispensable. To check this further, GM-CSF was omitted from the mix, and the effects of omitting one or two further cytokines (except IL-1 β and TNF- α) was examined (Fig. 3). As predicted from Fig. 2, the further omission of IL-4 had no effect on cluster formation. IL-4 was omitted from subsequent cytokine cocktails. Fig. 3 confirms the data of Fig. 2 showing that the omission of IL-7 in the absence of GM-CSF now had a marked effect on cluster formation, although interestingly this drop was less when other cytokines, in particular IL-4, were absent. Of the remaining cytokines, IL-3 appeared in these experiments to have the least influence, but it was retained in subsequent studies to maintain maximal DC cluster formation.

Effect of Antibody Against GM-CSF on Development of DC Clusters. The lack of any requirement for GM-CSF in the production of DC was surprising, in view of its requirement for DC outgrowth in other systems. It was possible

% CLUSTERS FORMED

-IL3	82 \pm 9	Control			
		IL1 β +IL3+IL4+IL7+SCF+TNF α			
-IL4	71 \pm 6	96 \pm 17			
-IL7	45 \pm 11	86 \pm 11	22 \pm 10		
-SCF	83 \pm 12	80 \pm 14	32 \pm 15	59 \pm 16	
	-IL3	-IL4	-IL7	-SCF	

Figure 3. The effects of omitting one or two cytokines from an initial mix of six on the formation of DC clusters. The experiment was similar to that described in Fig. 1, except that GM-CSF was not included in the initial mix, and that both IL-1 β and TNF- α were maintained as constant components.

the low CD4 precursors themselves, or some trace contaminant, produced sufficient endogenous GM-CSF. To test this possibility, a neutralizing antibody against GM-CSF was added to the cultures when they were initiated at a level known to block GM-CSF-dependent colony formation in culture. The cultures were stimulated by the above "optimal" mix of five cytokines, lacking GM-CSF. However, there was no significant drop in the number of DC clusters formed, nor any reduction in the apparent size of the clusters, when the antibody was added (Table 1). In view of the possibility that IL-3 was substituting for GM-CSF, because they share a common receptor chain (33), the test was repeated with both IL-3 and GM-CSF omitted from the cytokine mix. Again, the anti-GM-CSF had no significant effect in these relatively high density cultures.

Cytokine Requirements in Low Cell Density Cultures. Finally, to verify the requirement for all five cytokines, some simpler combinations were tested, at a lower precursor cell input

Table 1. The Effects of Antibody Against GM-CSF on the Generation of DC Clusters by Cultured Thymic Low CD4 Precursors

Cytokine mix	Anti-GM-CSF	Clusters per well
TNF- α + IL-1 β + IL-7 + SCF + IL-3	-	20 \pm 2
	+	18 \pm 2
TNF- α + IL-1 β + IL-7 + SCF	-	22 \pm 2
	+	19 \pm 1

Purified thymic low CD4 precursors were cultured at 3,000 cells per well in 0.01 ml medium for 4 d with the cytokines listed. Full details are given in Materials and Methods. Results are the means \pm SEM of pooled data from three experiments, each with five cultures per assay.

Table 2. The Effects of Various Cytokine Combinations on the Generation of DC Clusters in Low-Density Cultures of Thymic Precursors

Cytokines	Clusters per culture
No cytokines	0 ± 0
IL-1β	0 ± 0
TNF-α	0 ± 0
TNF-α + IL-1β	0 ± 0
IL-1β + TNF-α + SCF	0 ± 0
IL-1β + TNF-α + IL-3	0.4 ± 0.6
IL-1β + TNF-α + IL-7	0.1 ± 0.2
IL-1β + TNF-α + IL-7 + SCF	0.1 ± 0.2
IL-1β + TNF-α + IL-7 + IL-3	0.2 ± 0.4
IL-1β + TNF-α + SCF + IL-3	0.4 ± 0.6
IL-7 + SCF + IL-3	0.1 ± 0.1
IL-1β + TNF-α + IL-7 + SCF + IL-3	2.0 ± 0.9

Cultures of 250 low CD4 precursors were set up on 0.01 ml medium in Terasaki tray wells. The incidence of DC clusters was determined after 4 d of culture. Results are the means ± SEM of data pooled from two experiments, each with 20 cultures per condition.

(250 precursors per culture) to reduce any effects of endogenous growth factor production. All simpler cytokine combinations gave fewer DC clusters, or no clusters at all (Table 2), and the few clusters that were obtained appeared smaller. Two aspects of these low cell density cultures were notable. First, neither DC cluster formation nor cell expansion was evident in cultures with IL-1β alone or TNF-α alone, or IL-1β plus TNF-α, even though these cytokines were essential for the growth of DC in the cytokine mix. However, in the cultures with IL-1β alone, or in cultures with IL-1β plus TNF-α, ~20% of the individual, nonclustered surviving cells acquired dendritic morphology, suggesting IL-1β alone promoted some direct DC differentiation without cell division. In accordance with this, a few DC clusters were obtained when very high density cultures were incubated in IL-1β plus TNF-α (data not shown). A second aspect of the sparse cultures was the degree of dependence on IL-3 for DC cluster formation. In contrast to the dense cultures where omission of IL-3 had a smaller and variable effect (Figs. 2 and 3; Table 1), omission of IL-3 from the sparse cultures caused a much greater drop in both DC cluster formation and cell proliferation. A low level of endogenous IL-3 production by the precursor cells themselves is one possible explanation for this difference.

Under these low cell density culture conditions with the five cytokine mix, a net increase of the cells in the cultures was obtained, with growth extending longer and reaching three to four times the original cell input (Fig. 4). Over 90% of the cells harvested at days 3 to 4 of culture had dendritic morphology.

The Effect of CD40 Ligation and Flt3 Ligand Addition on DC Development. While our studies were in progress, two

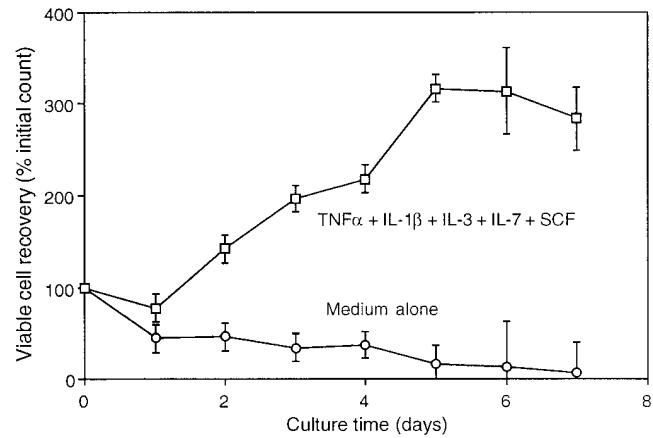


Figure 4. The growth of purified thymic low CD4 precursors under the influence of a mixture of five cytokines. Full details are given in Materials and Methods. The mix of five cytokines used was IL-1β, TNFα, IL-3, IL-7, and SCF, omitting the IL-4 and GM-CSF used in the original mix. The precursors (250) were cultured in 0.01 ml medium in Terasaki tray wells. Cell counts were performed after harvest in a hemocytometer with viability assessed by appearance under phase-contrast microscopy. Values are means ± SEM of pooled data from three experiments, each with 20 cultures per point.

further stimuli of DC development were reported. Soluble CD40L has been found to enhance DC survival and differentiation (34; for review see reference 12). Although it was ineffective alone, we found it enhanced the DC development stimulated by our previous five-cytokine mix. In the presence of soluble CD40L, the cultured cells more rapidly attained the extreme DC form with extended dendrites, the number of clusters and their size was increased, the cell yield increased, and fewer cells were found outside the clusters. The impression was of enhanced differentiation with an earlier peak of DC production. Very similar results, but more reproducible in the extent of the effect, were obtained by adding the mAb FGK45.5, reactive with CD40. This mAb was used instead of soluble CD40L in the experiment shown in Fig. 5 and subsequent experiments.

Recent studies at Immunex (35) have demonstrated that Flt3L injected into mice induces a striking increase in the levels of all types of DC in mouse lymphoid organs. Although Flt3L was without effect on the low CD4 precursors alone, when added together with the previous five-cytokine mix it enhanced DC development in culture. The number of cells produced in the cultures increased substantially and peaked earlier, whereas the clusters increased a little in both number and size (Fig. 5). The progeny cells again had DC morphology, although of a less extreme form than with CD40L.

The addition of both Flt3L and the mAb ligating CD40 to the cytokine mix of TNF-α, IL-1β, IL-3, IL-7, and SCF appeared to produce the optimal yield and morphological form of DC from the cultured low CD4 precursors, although these two additional "cytokines" were ineffective if used alone. With this new seven-"cytokine" mix, the numbers of cells produced from the thymic precursors

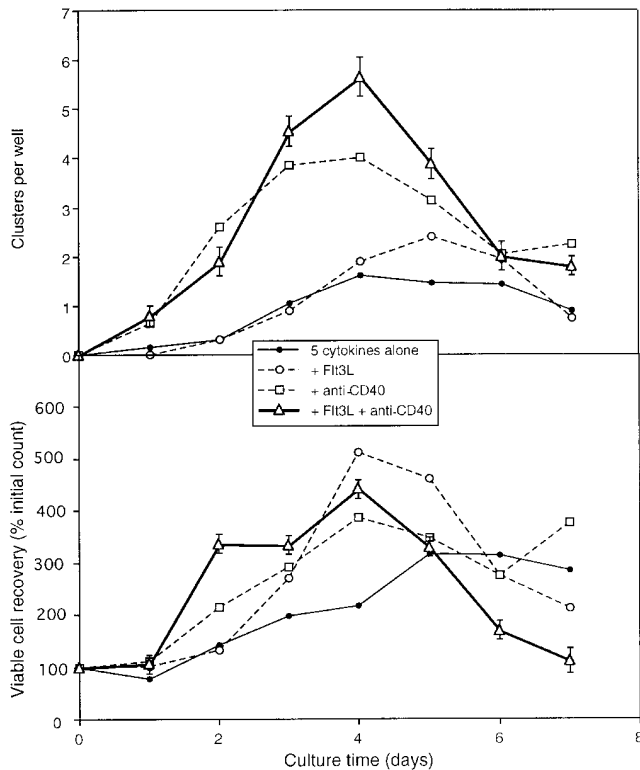


Figure 5. The effect of addition of Flt3L and mAb against CD40 on the growth and development of DC clusters from thymic low CD4 precursors. Cultures of 250 purified low CD4 precursors were set up in 0.01 ml medium in Terasaki tray culture wells. The finer continuous line gives the results with the basic cytokine mix (TNF- α , IL-1 β , IL-3, IL-7, SCF [●]). The two broken lines give the results of addition of Flt3L (○) or of the mAb FGK45.5 reactive with CD40 (□). The heavy line gives the results of adding both these reagents (Δ). Results are the means of pooled data from three to four experiments, each with 20 cultures per point. For clarity, SEM are only given for one set of data; the errors were similar in the other sets. Statistically significant increases over the results obtained with the basic five cytokine mix were obtained at day 4 of culture by adding Flt3L (cells, $P < 0.001$), by adding anti-CD40 (cells, $P < 0.001$; clusters, $P = 0.01-0.001$) and by adding both anti-CD40 and Flt3L (cells, $P < 0.001$; clusters, $P < 0.001$).

reached four- to fivefold the initial input by day 4 of culture (Fig. 5); this was a minimal estimate, because under these conditions the clusters were difficult to completely dissociate even with EDTA. The number of clusters was also maximized, and peaked at day 4, with this mix (Fig. 5). The appearance of the clusters is shown in Fig. 6. Over 95% of the individual cells harvested and recovered from such cultures had the morphological appearance of DC, all having multiple fine cytoplasmic extensions and many having more obvious "dendrites," as illustrated in Fig. 6.

To provide a comparison with the thymic precursors, a population of bone marrow precursors was also cultured with this seven-"cytokine" mix. The bone marrow precursors were selected by the same procedure used for the thymic precursors to produce a c-kit⁺Thy-1^{low} lineage marker-negative, light density, nonadherent population; however, there was no evidence that this was a homogenous precursor population. Growth from this bone marrow population

was rapid and extensive, reaching 10 times the initial cell input number by day 4. The resultant cell population was of mixed phenotype and included myeloid cells and early B cells. DC and DC clusters were produced, but comprised only 24% of the total cell yield; the net DC production was about half that obtained from the thymic precursors.

Production of DC in Culture by Thymic Low CD4 Precursors from GM-CSF Null Mice. The surprising lack of any requirement for GM-CSF in DC generation required a more critical assessment. It was possible that traces of endogenously derived GM-CSF persisted in the cultures despite the antibody-blocking experiments of Table 1. It was also possible that GM-CSF was required for the generation of the precursors from multipotent stem cells rather than at the later DC developmental steps reflected in our cultures. Accordingly, we assessed the development in culture of the thymic low CD4 precursors derived from GM-CSF "null" mice, with the GM-CSF gene deleted by homologous recombination (30). The yield of low CD4 precursors from the thymus of the GM-CSF "null" mice was similar to that obtained from the normal C57BL/6 control mice, indicating that the generation of this precursor population was independent of GM-CSF. When cultured at low cell density with the final complement of seven "cytokines" as in Fig. 5 (lacking GM-CSF), the thymic precursors from the GM-CSF null mice showed extensive proliferation and produced DC clusters, with >90% of the product cells showing typical dendritic morphology (Table 3). However, both the total cell expansion and the number of DC clusters was only 70–75% that obtained by culturing the same number of low CD4 precursors from normal mice. Thus, although DC development occurred efficiently in the absence of GM-CSF, it remained possible that traces of endogenous GM-CSF could enhance this development. Alternatively, GM-CSF in the normal mice may have slightly "conditioned" the precursors for an enhanced response, or removed some irrelevant cells from the "low CD4 precursor" pool.

Effects of IL-3 and GM-CSF on DC Development in Low Cell Density Cultures. The results with the GM-CSF null mice suggested that GM-CSF might have some stimulatory effect in low-density cultures, despite its lack of effect in the earlier high precursor cell input studies. Another possibility was that the requirement for GM-CSF was being largely met by the added IL-3, via interaction with a common receptor β chain; because the requirement for IL-3 only became pronounced in low-density cultures (Table 2), it was important to recheck this issue under these conditions. Accordingly, low CD4 precursors were cultured with IL-1 β , TNF- α , IL-7, SCF, Flt3L, and anti-CD40 mAb, and then the effects of adding IL-3 and/or GM-CSF were examined (Table 4).

Precursor cell expansion and DC development occurred in the absence of both IL-3 and GM-CSF, with the vast majority of cultured cells having dendritic morphology and aggregating into clusters. However, the yield of both DC and DC clusters was about half that seen in the presence of IL-3. Therefore, GM-CSF could partially substitute for IL-3 under these conditions. However, GM-CSF did not syner-

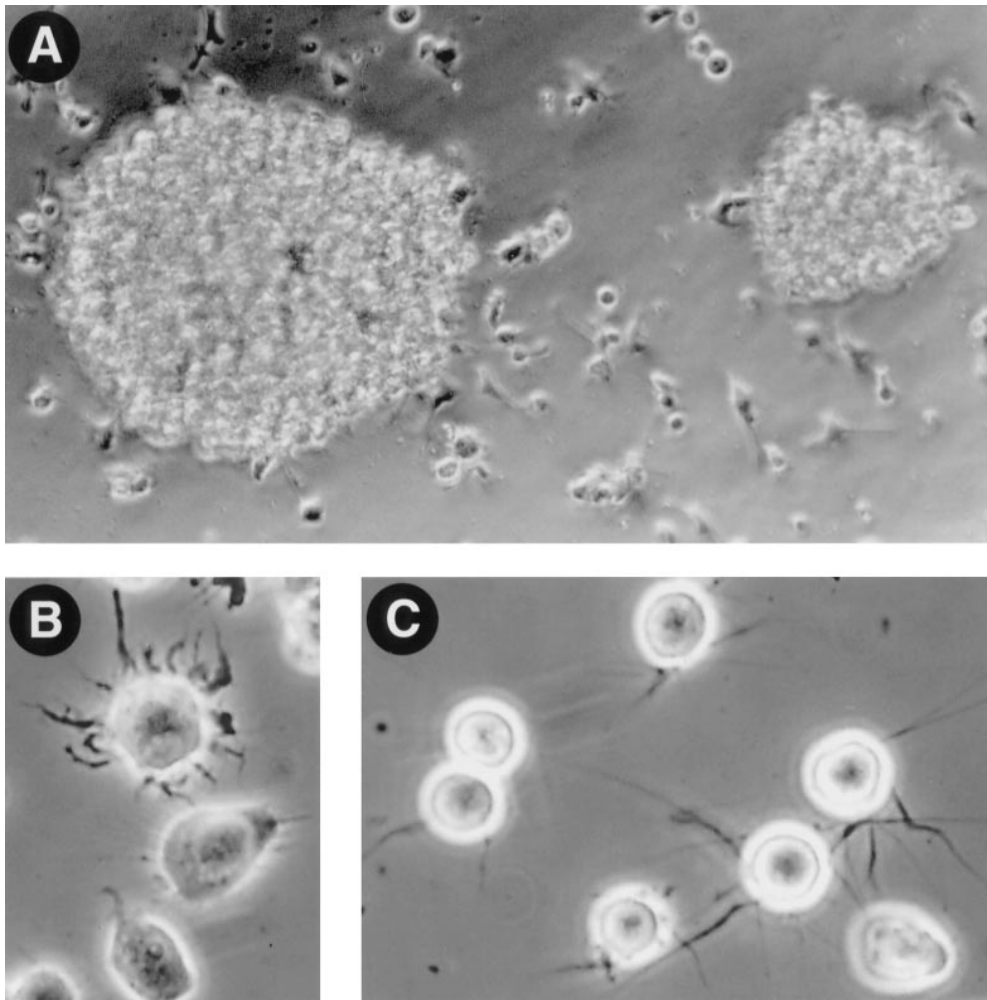


Figure 6. The clusters of DC and the individual DC developing in culture from thymic low CD4 precursors. The purified low CD4 precursors were cultured for 4 d in the presence of TNF- α , IL-1 β , IL-3, IL-7, SCF, Flt3L, and FGK45.5, the mAb reactive with CD40. The clusters (A) were photographed directly in the Terasaki tray cultures. The individual DC (B and C) were dissociated from the clusters using EDTA, replaced in culture medium in a slide chamber, and warmed for 2 h before photography. Almost all cells had multiple fine hairlike cytoplasmic extensions, only some of which are visible in the photographs. Original magnifications: (A) $\times 260$; (B and C) $\times 640$.

gize with IL-3, because some inhibition in cell expansion was noted when both were added together. Similar but slightly reduced effects were obtained when GM-CSF was added to the cultures at a 10-fold lower concentration.

The Surface Phenotype of the Cultured DC. Immunofluorescent staining and flow cytometry was used to analyze the surface antigens on the cultured cells. Figs. 7 and 8 give re-

sults for day 4 cultures of low CD4 precursors grown in the mix of TNF- α , IL-1 α , IL-3, IL-7, and SCF, together with the results for cultures grown in these five cytokines together with Flt3L and the anti-CD40 mAb. The surface phenotype of the DC was similar under these two conditions. It was also largely unchanged when day 6 cultures were compared with day 4 cultures.

Of the characteristic T cell markers, the cells from these cultures lacked CD4, CD8 α , CD8 β (not shown), CD3, and CD25. This implied they had lost the low level of CD4 characteristic of the precursors, but had not progressed to the next downstream CD25⁺ precursor, nor had they formed mature T cells. Surprisingly, they had not gained CD8 α , a marker characteristic of most thymic DC. The cells were Thy 1 positive, showing a wide range of Thy 1 expression; however, because many types of cultured cells express Thy 1, this marker is not useful for defining T lineage cells in culture.

Of the B lymphocyte lineage markers, the cells lacked B220, and also lacked BP-1, an early B cell marker expressed on thymic DC. However, BP-1 is known to be induced on DC by the thymic environment, and the low CD4 precursors produce BP-1⁻ DC if allowed to develop in the spleen (27).

Table 3. *The Growth and Development of DC Clusters from Thymic Low CD4 Precursors from GM-CSF "Null" Mice*

	Control C57BL/6 precursors	GM-CSF null precursors
Cells per culture	1,002 \pm 109	721 \pm 69
Clusters per culture	5.7 \pm 1.6	4.7 \pm 1.6

Purified low CD4 precursors, 250 per 0.01 ml medium in Terasaki culture wells, were cultured for 4 d in the presence of TNF- α , IL-1 β , IL-3, IL-7, SCF, Flt3L, and FGK45.5 mAb against CD40. Results are the means \pm SEM of pooled data from two experiments, each with 20 cultures per determination.

Table 4. *The Influence of GM-CSF and IL-3 on DC Development from Low CD4 Thymic Precursors in Low-Density Cultures*

Cytokines	Day 4	
	Cells per culture	Clusters per culture
IL-1 β , TNF- α , IL-7, SCF, Flt3L, anti-CD40 alone	4,930	1.8 \pm 0.4
IL-1 β , TNF- α , IL-7, SCF, Flt3L, anti-CD40 plus IL-3	10,130	4.3 \pm 0.4
IL-1 β , TNF- α , IL-7, SCF, Flt3L, anti-CD40 plus GM-CSF	6,570	3.7 \pm 0.4
IL-1 β , TNF- α , IL-7, SCF, Flt3L, anti-CD40 plus IL-3 and GM-CSF	8,360	4.6 \pm 0.5

Purified low CD4 thymic precursors were cultured at 250 cells per well in 0.01 ml medium for 4 d with the cytokines listed. Results are the pooled data from two experiments, each with 20 cultures per condition. Cluster counts are the means \pm SEM of individual direct culture counts. Cell counts were performed on the pool of the 20 cultures in each experiment after harvest, and the results are the mean of the two experiments.

Of the macrophage/granulocyte markers, the cells were negative for Gr-1. They stained at levels varying from negative to moderately positive for CD11b (Mac-1) and negative to moderately positive for F4/80. The very strong staining characteristic of macrophages was not seen with either marker. CD11b is expressed at levels ranging from low to high on different lymphoid tissue DC (26, 27, 35). F4/80 has been observed on cultured DC precursors (13), but is not normally expressed at this level on mature tissue DC (26).

Of the typical DC markers, the cultured cells expressed very high levels of class I and class II MHC, high levels of CD11c, and moderate levels of DEC205. They expressed B7-1 (CD80) and B7-2 (CD86), characteristics of mature DC. As do most DC, they expressed CD40, CD44, and HSA, the heat stable antigen (not shown); these markers are also found on other cell types. They also had the high forward and side scatter characteristic of DC, as expected from their size and appearance in Fig. 6. Overall they resembled mature DC, the only anomaly being the absence of CD8 α , a marker characteristic of thymic DC in the mouse but present on only about half of splenic or LN DC. It should be noted that the low CD4 precursors lack surface class II MHC, CD11c, DEC205, CD80, and CD86 (20, 21, 29), an observation we confirmed in this study (data not shown), so differentiation toward expression of these DC markers had occurred in the cultures.

T Cell Stimulatory Activity of the Cultured DC. To determine if the culture system produced functional DC, the DC produced from thymic precursors by culture in the presence of IL-1 β , TNF- α , IL-3, IL-7, SCF, Flt3L, and mAb binding CD40 were compared with freshly isolated thymic DC in their ability to stimulate the proliferation of allogeneic CD4 T cells. The small scale mixed leukocyte cultures used (28) involved 20,000 pure CD4⁺ LN T cells as responders, and small numbers of either pure thymic DC or cultured DC as stimulators; no exogenous cytokines were added. Both the DC cultured with the full seven-cytokine mix and the normal thymic DC were found to be efficient stimulators of mature T cell proliferation; both gave a proliferative response peaking at day 3 to 3.5, and both gave a good DC dose-response relationship and a

very high stimulation index (Fig. 9). The DC cultured with the full cytokine mix generally gave better T cell stimulation than freshly isolated thymic DC.

Frequency of Responding and DC-producing Precursors. The rapid increase in cell counts during the response of the low CD4 precursor preparation to the cytokine mixes (Figs. 4 and 5) suggested that a significant proportion of the cells were responding. However, because it required \sim 50 precursors to form one DC cluster at day 4, it may have been that only 2% of the precursor population was committed to DC development. The argument against this was that the incidence of cells with DC morphology was very much higher than 2% early in the culture, and that the clusters represented aggregates rather than colonies derived from a single precursor. To determine the actual incidence of responding cells, a series of experiments was conducted in which the low CD4 precursors were set up in Terasaki tray cultures at the 1-cell-per-well level in medium containing the optimum mix of "cytokines" (IL-1 β , TNF- α , IL-3, IL-7, SCF, Flt3L, and anti-CD40). After 2 h, the cultures were examined under inverted phase microscopy, and cultures with a single precursor in the well were selected for further day-by-day observation. In one such experiment the fate of 27 single precursor cells was followed over 3 d. By day 1, nine of the starting cells had died, but all but one of the remaining viable cells showed a clear DC morphology; of these 70% survivors with DC morphology, 20% had already undergone one cell division. By day 3, one further culture had terminated in cell death, but all the remainder had divided to produce between 2 and 10 progeny; 90% of the viable progeny cells in these clones were of DC morphology. In some cases, the progeny cells stayed associated as a minicluster, but in many cultures they moved apart. Two subsequent experiments confirmed these observations. Thus the majority (at least 70%) of the cells in the low CD4 precursor preparation could be considered as potential DC precursors. We are unable to assess whether the 30% of precursors that died in our cultures were also potential DC precursors, or whether these cells were committed to another lineage. It was of interest that differentiation of these low CD4 precursors to a DC morphology was rapid and usually preceded cell division.

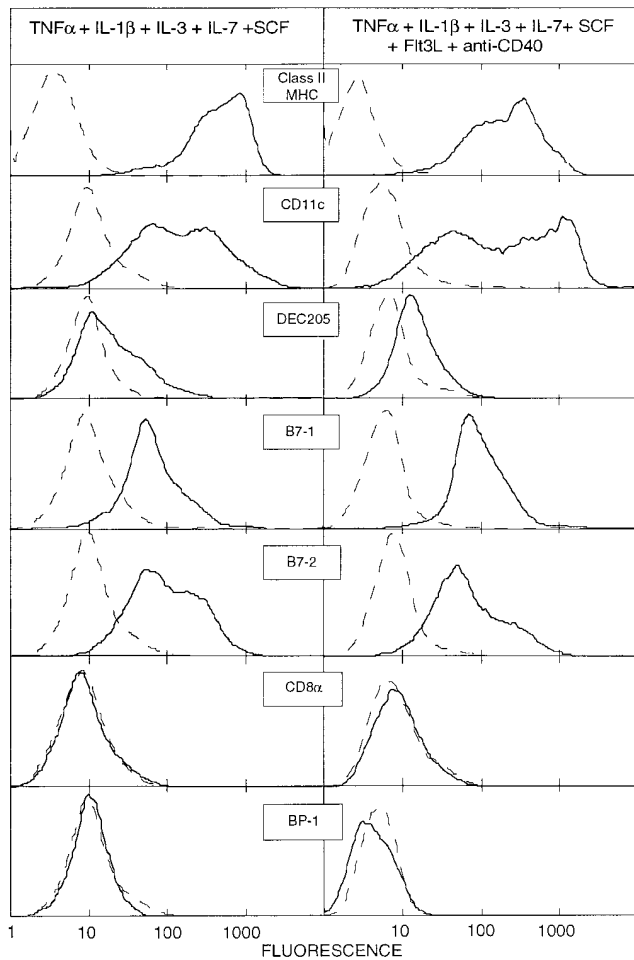


Figure 7. The expression of typical thymic DC surface markers on the DC developing in culture of thymic low CD4 precursors. The precursors (20,000) were grown for 4 d in 0.1 ml medium in 96-well, flat-bottomed culture trays, in the presence of IL-1 β , TNF- α , IL-3, IL-7, and SCF, alone (*left*) or together with Flt3L and the mAb FGK45.5 reactive with CD40 (*right*). The cultured were harvested, pooled, dissociated by EDTA treatment, and then stained with mAb, generally in two fluorescent colors. Full details are given in Materials and Methods. Dead or damaged cells, ~15% of the total, were excluded from analysis by propidium iodide staining and forward light scatter characteristics; otherwise, all cells growing in the cultures are represented in fluorescence distribution profiles. The broken line gives the background staining, omitting the relevant mAb. Each result is representative of two to five such analyses.

Discussion

The growth in culture of a highly purified early thymic T precursor population, the low CD4 precursor, has led in these experiments to the development of DC rather than T cells. Because on intravenous transfer to irradiated recipients this same population forms T cells, B cells, NK cells, and DC (18–23; for review see reference 24), only one of these potentials has been realized under our culture conditions. We cannot at present determine whether all the cells in this precursor population have all four developmental potentials, because we are at present unable to produce T cells, B cells or NK cells from these precursors in culture. However, because at least 70% of these cells, originally se-

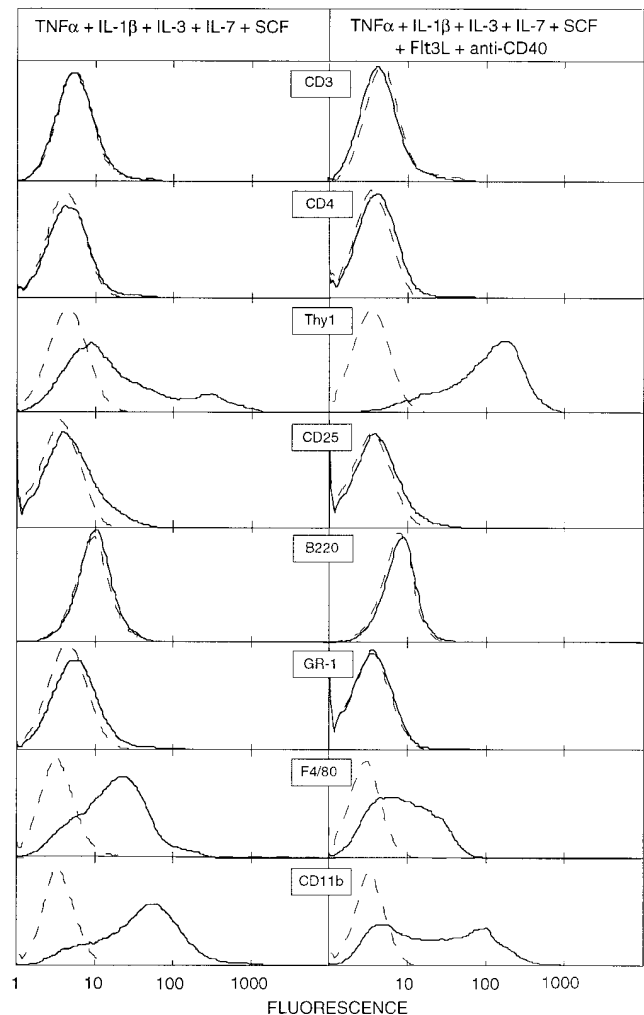


Figure 8. The expression of characteristic T cell, B cell, or myeloid cell markers on the DC developing in culture of thymic low CD4 precursors. Details are as in Fig. 7.

lected as being precursors of T cells, have the potential to develop into DC, we consider it likely that the T lymphocyte precursors and the DC precursors are identical. It seems highly unlikely that the DC arise from a 70% myeloid precursor “contaminant” in the T precursor population because there was no detectable myeloid response in transfer experiments (19), and the incidence of cells able to respond to GM-CSF by forming colonies or proliferating in culture is $\leq 1\%$ (19, 29). The results support the view that thymic DC are of lymphoid precursor origin.

The cytokine mix we have used to produce DC is totally different from those usually used to produce DC in culture, and it is much more complex. Some of this complexity may be attributed to our use of a pure precursor population, because there are no other cells around to contribute their cytokine products to the developing DC precursor. Apart from the number of cytokines involved, the most striking aspect of our study was our difficulty in demonstrating any marked effect of GM-CSF on the development of DC from the thymic precursors. This stands in complete

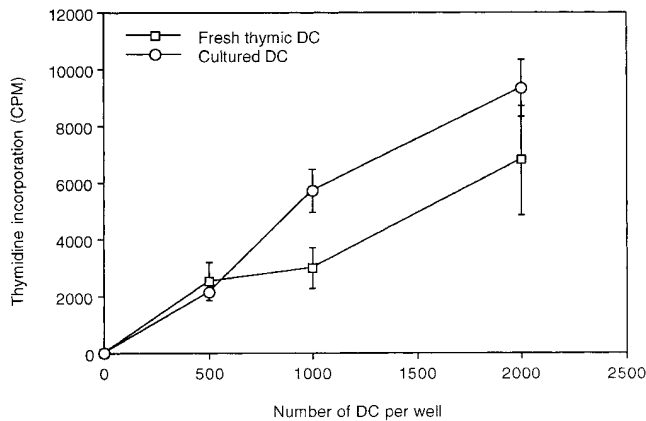


Figure 9. The stimulation of CD4 T cell proliferation by the DC derived in culture from the thymic low CD4 precursors. The cultured DC were harvested on day 4 from cultures of thymic low CD4 precursors grown in the presence of the cytokines IL-1 β , TNF- α , IL-3, IL-7, SCF, Flt3L, and the mAb FGK45.5 reactive with CD40. These were compared with normal thymic DC extracted directly from the thymus and finally purified by sorting based on CD11c expression. Purified CBA LN CD4 T cells (20,000) were cultured for 3 d with various levels of the C57BL/6-derived DC, and then the cultures were pulsed for 9 h with [3 H]TdR. The cells were collected onto glass-fiber filters, and proliferation was assessed by measuring incorporated radioactivity using gas flow scintillation counting. Full details are given in Materials and Methods. Results are the means \pm SEM of the pooled data from two experiments, each with five cultures per point. Similar results but with somewhat lower counts were obtained at days 2.5 and 3.5 of harvest. The background count with T cells alone was 17 ± 1 cpm, and the stimulation index was >300 . The background count with 2,000 fresh thymic DC alone was 77 ± 22 cpm, and with 2,000 cultured DC alone was 109 ± 14 cpm.

contrast to the central role of this cytokine in most studies of DC development in culture (2–4, 6–17). Our experiments using anti-GM-CSF antibody, and using GM-CSF-deficient mice, indicate that this is not due to endogenous GM-CSF generation nor to a requirement for GM-CSF at an earlier developmental stage than the low CD4 precursor. Nor can it be entirely explained by a substitution of IL-3 for GM-CSF as the cytokine signaling through the GM-CSF receptor β chain, although GM-CSF and IL-3 are partially exchangeable in giving a twofold enhancement of DC production. The thymic precursors and their thymic DC progeny presumably have receptors for GM-CSF, because this cytokine when used alone does improve viability in culture, and in the absence of IL-3 it can enhance DC production; however, it is not essential in cell proliferation or differentiation to DC. It is possible that the role of GM-CSF in all other studies to date has been indirect, via its cytokine inductive effects on cells other than the DC precursors in the heterogeneous precursor preparations. However, it seems more likely that this lack of effect of GM-CSF is due to our outgrowth of a lymphoid-derived rather than a myeloid-derived class of DC. It should be noted that the evidence for a myeloid origin of DC comes from cultures grown using GM-CSF, so this type of experiment would selectively address the origin of only one type of DC. It will now be important to determine what proportion of DC found in various lymphoid organs are of mye-

loid precursor versus lymphoid precursor origin. It is of interest that DC can also be produced from bone marrow precursors using our cytokine mix lacking GM-CSF. However we do not know the lineage origin of these DC.

The DC we develop in culture from the thymic low CD4 precursors using the optimum complement of cytokines appear similar to normal thymic DC in CD4 T cell stimulatory function, and in most of the common DC cell surface markers. However, there are some differences in surface phenotype from normal thymic DC. Some markers that are higher than on normal thymic DC may be a simple consequence of growth in culture; thus CD11b (Mac-1), although lower on freshly isolated normal thymic DC, may be upregulated by overnight incubation in culture to levels similar to those on the DC we generate in culture (D. Vrevec, unpublished data). Some markers that are missing may indicate that the factors required for upregulation, present in the normal thymus, are lacking in our cultures. Thus we have already established that BP-1, normally expressed on thymic DC and on the DC progeny of the low CD4 precursors if they develop in the thymus of an irradiated recipient, is absent when the same precursors produce DC progeny in the spleen (27). We assume, therefore, that our cultures lack the thymus-specific induction factors required for BP-1 expression. However, the absence of CD8 α on the DC we generate from the low CD4 precursors in culture is surprising and presents us with a paradox.

Most DC in the thymus, and a proportion of those in spleen and LN, express CD8 as an $\alpha\alpha$ homodimer (21, 26). We have found in intravenous transfer studies that CD8 α serves as a marker of the DC progeny of the low CD4 precursor, in both the thymus and the spleen of irradiated recipients (27). On this basis we suggested that normal CD8 α^+ DC are lymphoid derived, whereas the CD8 $^-$ DC found in spleen and LN are myeloid derived. It now seems CD8 α is not an inevitable marker of lymphoid-derived DC, raising the possibility that many of the CD8 $^-$ DC found in normal lymphoid tissue are also lymphoid derived. In fact, in more recent studies (L. Wu, unpublished), we have found the DC progeny found in LN after intravenous transfer of thymic low CD4 precursors include CD8 $^-$ as well as CD8 $^+$ DC. CD8 α expression may be induced by some aspect of the environment common to both thymus and spleen, less pronounced in LN, but not reproduced at all in our cultures.

DC expanded in culture using GM-CSF show great promise as natural adjuvants for enhancing immune responses to tumors and other antigens (for review see reference 36). Our results indicate that the use of different combinations of cytokines could allow the outgrowth of different types of DC, originating from different precursor cells. Our other studies indicate that some types of DC have an inhibitory or regulatory effect on T cell responses (28, 37). Accordingly, it will be important to reevaluate the immunostimulatory capacity of the DC produced when different cytokine combinations are used, because it may be possible to generate DC that inhibit rather than enhance an immune response.

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