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 CD8α 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 CD8α, 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).
These thymic precursors also formed CD8α+ DC in the spleen after intravenous transfer, suggesting that the CD8α+ 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. The GM-CSF–null mice, produced at the Ludwig Institute (30), were originally on a C57BL/6 × 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 5–7 wk-old female CBA/J mice bred under specific pathogen–free conditions at 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 isosmotic with mouse serum. The light-density cells were then coated with a cocktail of mAbs reactive with CD3, CD4, Thy 1, CD25, B cell antigen B220, erythrocyte antigen TER119, granulocyte antigen Gr-1, macrophage antigen F480, FeR1I1 and CD11b, and then the coated cells were removed using anti-Ig–coated magnetic beads. Finally, the 1% remaining nonadherent cells were selected 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 homogenous 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 isosmotic 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-1β (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 Immunoex Corp. Flt3/Flik2 ligand (Flt3L), 100 ng/ml, and antibody against GM-CSF, 2 μg/ml, was provided by Dr. N. Nicola (The Walter and Eliza Hall Institute). CD40 ligand (CD40L) and mAb against CD40, FGK45.5, 1 μg/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; CD8α, biotin–conjugated 53-6.7; CD8β, biotin–conjugated 53-5.8; CD3, PE-conjugated KT3-1.1; class I MHC, biotin–conjugated M1/42; class II MHC, Texas red–conjugated
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]ThdR. 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-1β, 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,
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-1β 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-1β 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-1β 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 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-1β + IL-7 + SCF + IL-3 | - | 20 ± 2 |
|                   | +           | 18 ± 2 |
| TNF-α + IL-1β + IL-7 + SCF | - | 22 ± 2 |
|                   | +           | 19 ± 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 ± SEM of pooled data from three experiments, each with five cultures per assay.
While our studies were in progress, two DC Development.

90% of the cells harvested at days 3 to 4 of culture had den-

three to four times the original cell input (Fig. 4). Over

five cytokine mix, a net increase of the cells in the cultures

endogenous IL-3 production by the precursor cells them-

cluster formation and cell proliferation. A low level of

were incubated in IL-1

DC clusters were obtained when very high density cultures

tation without cell division. In accordance with this, a few

were incubated in IL-1

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, nonclus-
tered surviving cells acquired dendritic morphology, sug-
gest ing IL-1β alone promoted some direct DC differen-
tation 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 de-

pendence 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 them-
selves 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 den-
dritic morphology.

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

further stimuli of DC development were reported. Soluble

CD40L has been found to enhance DC survival and differ-
etiation (34; for review see reference 12). Although it was

ineffective alone, we found it enhanced the DC develop-
ment 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 ob-
tained by adding the mAb FGK45.5, reactive with CD40.

This mAb was used instead of soluble CD40L in the exper-
iment 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. Al-

though 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 substan-
tially 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 morpho-

logical 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

(250 precursors per culture) to reduce any effects of endog-

enous growth factor production. All simpler cytokine com-
binations gave fewer DC clusters, or no clusters at all (Ta-

ble 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 expan-

sion 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.

Second, the cell numbers of cells produced from the thymic

precursors

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 + SCF + IL-3 | 0.2 ± 0.4            |
| IL-7 + SCF + IL-3          | 0.4 ± 0.6            |
| IL-1β + TNF-α + IL-7 + SCF | 0.1 ± 0.1            |
| IL-1β + TNF-α + IL-7 + SCF | 2.0 ± 0.9            |

Figure 4. The growth of purified thymic low CD4 precursors under
the influence of a mixture of five cytokines. Full details are given in Ma-
terials 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.

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reached four- to fivetfold 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-1low 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).

Effect 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-1β, 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-
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 results for day 4 cultures of low CD4 precursors grown in the mix of TNF-α, IL-1β, 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) ×260; (B and C) ×640.

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 ± 109               | 721 ± 69              |
| Clusters per culture | 5.7 ± 1.6                 | 4.7 ± 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-1β, IL-3, IL-7, SCF, Flt3L, and FGK45.5 mAb against CD40. Results are the means ± SEM of pooled data from two experiments, each with 20 cultures per determination.
DC markers had occurred in the cultures. It should be noted that the low CD4 precursors lack surface expression of CD4, CD11c, and moderate levels of DEC205. They expressed very high levels of class I and class II MHC, high levels of B7-1 (CD80) and B7-2 (CD86), characteristics of mature DC. The argument against this was that the incidence of cells with DC morphology was very much higher than freshly isolated thymic DC.

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 CD8α, 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-1β, TNF-α, IL-7, SCF, Flt3L, anti-CD40 alone, or IL-1β, TNF-α, IL-7, SCF, Flt3L, anti-CD40 plus GM-CSF 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.

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

| Cytokines                                      | Cells per culture | Clusters per culture |
|------------------------------------------------|-------------------|----------------------|
| IL-1β, TNF-α, IL-7, SCF, Flt3L, anti-CD40 alone | 4,930             | 1.8 ± 0.4            |
| IL-1β, TNF-α, IL-7, SCF, Flt3L, anti-CD40 plus IL-3 | 10,130            | 4.3 ± 0.4            |
| IL-1β, TNF-α, IL-7, SCF, Flt3L, anti-CD40 plus GM-CSF | 6,570             | 3.7 ± 0.4            |
| IL-1β, TNF-α, IL-7, SCF, Flt3L, anti-CD40 plus IL-3 and GM-CSF | 8,360             | 4.6 ± 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 ± 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.
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 selected 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 ≤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
The cytokines IL-1, 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 [3H]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 ± 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.

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-1β, 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 [3H]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 ± 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.

Most DC in the thymus, and a proportion of those in spleen and LN, express CD8 as an αα homodimer (21, 26). We have found in intravenous transfer studies that CD8α 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 CD8α+ DC are lymphoid derived, whereas the CD8α- DC found in spleen and LN are myeloid derived. It now seems CD8α is not an inevitable marker of lymphoid-derived DC, raising the possibility that many of the CD8α DC found in normal lymphoid tissue are also lymphoid derived. In fact, in more recent studies (L. Wu, unpublished), we have found the CD8α progeny found in LN after intravenous transfer of thymic low CD4 precursors include CD8α+ as well as CD8α+ DC. CD8α 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 immune-stimulatory 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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References

1. Steinman, R.M. 1991. The dendritic cell system and its role in immunogenicity. Annu. Rev. Immunol. 9:271–296.
2. Reid, C.D.C., P.R. Fryer, C. Clifford, A. Kirk, J. Tikerpae, and S.C. Knight. 1990. Identification of hematopoietic progenitors of macrophages and dendritic Langerhans cells (DL-CFU) in human bone marrow and peripheral blood. Blood. 76:1139–1149.
3. Inaba, K., R.M. Steinman, M. Witmer Pack, H. Aya, M. Ikehara, S. Muramatsu, and R.M. Steinman. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. J. Exp. Med. 173:1157–1167.
4. Inaba, K., M. Inaba, N. Romani, H. Aya, M. Deguchi, S. Ikehara, S. Muramatsu, and R.M. Steinman. 1992. Generation of proliferating dendritic cell precursors in mouse blood. J. Exp. Med. 175:1157–1167.
5. Caux, C., C. Dezutter-Dambuyant, D. Schmitt, and J. Banchereau. 1992. GM-CSF and TNF-α cooperates in the generation of dendritic Langerhans cells. Nature (Lond.). 360:258–260.
6. Scheicher, C., M. Mehlig, R. Zecher, and K. Reske. 1992. Dendritic cells from mouse bone marrow: in vitro differentiation using low doses of recombinant granulocyte-macrophage colony-stimulating factor. J. Immunol. Methods. 154:253–264.
7. Reid, C.D.L., A. Stackpole, A. Meager, and J. Tikerpae. 1992. Interactions of tumor necrosis factor with granulocyte-macrophage colony-stimulating factor and other cytokines in the regulation of dendritic cell growth in vitro from early bi-potent CD34+ progenitors in human bone marrow. J. Immunol. 149:2681–2688.
8. Santiago-Schwarz, F., N. Divaris, C. Kay, and S.E. Carsons. 1993. Mechanisms of tumor necrosis factor-granulocyte-macrophage colony-stimulating factor-induced dendritic cell development. Blood. 82:3019–3029.
9. Romani, N., S. Gruner, D. Brang, E. Kampgen, A. Lenz, B. Trockenbacher, G. Konwalinka, P.O. Fritch, R.M. Steinman, and G. Schuler. 1994. Proliferating dendritic cell progenitors in human blood. J. Exp. Med. 180:83–93.
10. Lu, L., J. Woo, A.S. Rao, Y. Li, S.C. Watkins, S. Qian, T.E. Starzl, A.J. Demetrius, and A.W. Thomson. 1994. Propagation of dendritic cell progenitors from normal mouse liver using granulocyte/macrophage colony-stimulating factor and their maturation development in the presence of type 1 collagen. J. Exp. Med. 179:1823–1834.
11. Strunk, D., K. Rappersberger, C. Egger, H. Strobil, E. Krömer, A. Elbe, D. Maurer, and G. Stingl. 1996. Generation of human dendritic cells/Langerhans cells from circulating CD34+ hematopoietic progenitor cells. Blood. 87:1292–1302.
12. Caux, C., and J. Banchereau. 1996. In vitro regulation of dendritic cell development and function. In Blood Cell Biochemistry, Vol. 7, Hemopoietic Growth Factors and their Receptors. A. Whetton and J. Gordons, editors. Plenum Press, London. In press.
13. Inaba, K., M. Inaba, M. Deguchi, K. Hagi, R. Yasumizu, S. Ikehara, S. Muramatsu, and R.M. Steinman. 1993. Granulocytes, macrophages, and dendritic cells arise from a common major histocompatibility complex class II-negative progenitor in mouse bone marrow. Proc. Natl. Acad. Sci. USA. 90:3038–3042.
14. Young, J.W., P. Szabolics, and M.A.S. Moore. 1995. Identification of dendritic cell colony-forming units among normal human CD34+ bone marrow progenitors that are expanded by c-kit ligand and yield pure dendritic cell colonies in the presence of granulocyte/macrophage colony-stimulating factor and tumor necrosis factor α. J. Exp. Med. 182:1111–1120.
15. Kabel, P.J., M. de Haan-Meulman, H.A. Voorbij, M. Kleingold, E.F. Knol, and H.A. Drexhage. 1989. Accessory cells with a morphology and marker pattern of dendritic cells can be obtained from elutriator-purified blood monocyte fractions. An enhancing effect of metrizamide in this differentiation. Immunobiology. 179:395–411.
16. Rossi, G., N. Heveker, B. Thiele, H. Gelderblom, and F. Steinbach. 1992. Development of a Langerhans cell phenotype from peripheral blood monocytes. Immunol. Lett. 31:189–198.
17. Sallusto, F., and A. Lanzavecchia. 1994. Efficient presentation of soluble antigen by cultured human dendritic cells maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor α. J. Exp. Med. 179:1109–1118.
18. Wu, L., R. Scollay, M. Argentor, M. Pearse, G.J. Spangrude, and K. Shortman. 1991. CD45 expressed on earliest T-lineage precursor cells in the adult murine thymus. Nature (Lond.). 349:71–74.
19. Wu, L., R. Scollay, M. Pearse, G.J. Spangrude, and K. Shortman. 1991. Developmental potential of the earliest precursor cells from the adult thymus. J. Exp. Med. 174:1617–1627.
20. Aradvin, C., L. Wu, C. Li, and K. Shortman. 1993. Thymic dendritic cells and T cells develop simultaneously within the thymus from a common precursor population. Nature (Lond.). 362:761–763.
21. Wu, L., D. Vremec, C. Ardavin, K. Winkel, G. Suss, H. Georgiou, E. Maraskovsky, W. Cook, and K. Shortman. 1995. Mouse thymus dendritic cells: kinetics of development.
and changes in surface markers during maturation. Eur. J. Immunol. 25:418–425.

22. Matsuzaki, Y., J. Gyotoku, M. Ogawa, S. Nishikawa, Y. Kat-sura, G. Gachelin, and H. Nakauchi. 1993. Characterization of c-kit positive intrathymic stem cells that are restricted to lymphoid differentiation. J. Exp. Med. 178:1283–1291.

23. Moore, T.A., and A. Zlotnik. 1995. T-cell lineage commitment and cytokine responses of thymic progenitors. Blood. 86:1850–1860.

24. Shortman, K., and L. Wu. 1996. Early T lymphocyte progenitors. Annu. Rev. Immunol. 14:29–47.

25. Galy, A., M. Travis, D. Cen, and B. Chen. 1995. Human T, B, natural killer and dendritic cells arise from a common bone marrow progenitor subset. Immunity. 3:459–473.

26. Vremec, D., M. Zorbas, R. Scollay, D.J. Saunders, C.F. Ar-davin, L. Wu, and K. Shortman. 1992. The surface phenotype of dendritic cells purified from mouse thymus and spleen: investigation of the CD8 expression by a subpopulation of dendritic cells. J. Exp. Med. 176:47–58.

27. Wu, L., C.-L. Li, and K. Shortman. 1996. Thymic dendritic cell precursors: relationship to the T-lymphocyte lineage and phenotype of the dendritic cell progeny. J. Exp. Med. 184:903–911.

28. Süss, G., and K. Shortman. 1996. A subclass of dendritic cells kills CD4 T cells via Fas/Fas–ligand–induced apoptosis. J. Exp. Med. 183:1789–1796.

29. Saunders, D., H.M. Georgiou, L. Wu, and K. Shortman. 1995. Induction of limited growth and differentiation of early thymic precursor cells by thymic epithelial cell lines. Immunol. Lett. 47:45–51.

30. Stanley, E., G.J. Lieschke, D. Grail, D. Metcalf, G. Hodgson, J.A.M. Gall, D.W. Maher, J. Cebon, V. Sinickas, and A.R. Dunn. 1994. Granulocyte/macrophage colony-stimulating factor-deficient mice show no major perturbation of hematopoiesis but develop a characteristic pulmonary pathology. Proc. Natl. Acad. Sci. USA. 91:5592–5596.

31. Winkel, K., F. Sotzik, D. Vremec, P.U. Cameron, and K. Shortman. 1994. CD4 and CD8 expression by human and mouse thymic dendritic cells. Immunol. Lett. 40:93–99.

32. Ismaili, J., M. Antica, and L. Wu. 1996. CD4 and CD8 expression and T cell antigen receptor gene rearrangement in early intrathymic precursor cells. Eur. J. Immunol. 26:731–737.

33. Nicola, N.A. 1994. Guidebook to Cytokines and their Receptors. Oxford University Press, Oxford, U.K. 261 pp.

34. Caux, C., C. Massacrier, B. Vanbervliet, B. Dubois, C. Van Kooten, I. Durand, and J. Banchereau. 1994. Activation of human dendritic cells through CD40 cross-linking. J. Exp. Med. 180:1263–1272.

35. Maraskovsky, E., K. Brasel, M. Teepe, E.R. Roux, S.D. Lyman, K. Shortman, and H.J. McKenna. 1996. Dramatic increase in the numbers of functionally mature dendritic cells in mice treated with Flt3 ligand: multiple dendritic cell subpopulations identified. J. Exp. Med. 184:1953–1962.

36. Young, J.W., and K. Inaba. 1996. Dendritic cells as adjuvants for class I major histocompatibility complex–restricted antitumor immunity. J. Exp. Med. 183:7–11.

37. Kronin, V., K. Winkel, G. Suss, A. Kelso, W. Heath, J. Kir-berg, H. von Boehmer, and K. Shortman. 1996. A subclass of dendritic cells regulates the response of naive CD8 T cells by limiting their IL-2 production. J. Immunol. In press.