# GRANULOCYTE/MACROPHAGE COLONY-STIMULATING FACTOR IS ESSENTIAL FOR THE VIABILITY AND FUNCTION OF CULTURED MURINE EPIDERMAL LANGERHANS CELLS

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Langerhans cells (LC)[1] are leukocytes that are distributed in the suprabasal region of squamous epithelia, particularly skin. LC undergo marked changes when epidermal cell suspensions are prepared by a standard trypsinization protocol and placed in culture for 1–3 d. Several cell surface markers (Fc receptors, the F4/80 antigen, membrane ATPase, and nonspecific esterase) decrease substantially (1, 2), while others (class I and II products of the MHC) increase (3). When accessory function for primary T-dependent immune responses is quantitated, the activity of the LC population increases some 10–30-fold (1, 4). Therefore LC seem to mature immunologically in bulk epidermal culture and acquire most of the features of lymphoid dendritic cells.

Because LC maturation leads to the development of accessory function for primary immune responses, this process may contribute to the sensitization phase of cell-mediated immunity. Here, we asked whether maturation is autonomous, or if exogenous cells and factors are required. We developed a panning technique to enrich the trace LC population from freshly dissociated mouse epidermis. When this was done, the viability and function of cultured LC was dependent upon factors that were found in the medium of keratinocyte cultures, as well as stimulated macrophages and T cells. We will describe these experiments and the identification of granulocyte/macrophage colony-stimulating factor (GM-CSF) as the principal if not exclusive mediator for the production of functioning LC.

## Materials and Methods

### Mice

(BALB/c × DBA/2)F₁ [(C × D2)F₁], A, B6.H-2k, (C57BL/6 × DBA/2)F₁ [(B6 × D2)F₁], 6–12 wk of age and of both sexes, were obtained from the Trudeau Institute, Saranac Lake, NY.

¹ Abbreviations used in this paper: GM-CSF, granulocyte/macrophage colony-stimulating factor; KCM, keratinocyte-conditioned medium; LC, Langerhans cell.

## LC Enrichment by Panning (5)

Epidermal cells were isolated from mouse ears exactly as described (1) except that 0.5% trypsin was used. Briefly, dorsal and ventral halves of ear skin were floated on a trypsin solution for 20 and 40 min, respectively, the epidermal sheets were peeled from the underlying dermis, and the sheets were dissociated by gentle shaking on a sieve. Ia$^+$ LC accounted for ~1% of the cell suspension and were enriched by the following procedure. Most Thy-1$^+$ keratinocytes (6) were removed by treating the suspension for 1 h in cytotoxicity medium (RPMI-1640 supplemented with 1% BSA and 25 mM Hepes buffer) at $3 \times 10^6$ cells/ml with mAb anti-Thy-1 (culture supernatant from clone 13.4, TIB 99 from the American Type Culture Collection [ATCC], Bethesda, MD) and complement (3-4-wk-old rabbit serum from Pel-Freeze, Rodgers, WI). 80-90% of the suspended cells became trypan blue–positive. To remove dead cells, the suspension was diluted in HBSS, centrifuged, and resuspended in 0.1% trypsin with 80 $\mu$g/ml DNase in HBSS at $10^6$ viable cells per milliliter. After 20 min at 37°C, the cells were diluted in RPMI-1640 with 10% FCS, centrifuged, and resuspended to $10^6$ cells/ml in an mAb that would selectively bind to the LC (~7-15% of the suspension at this point). For most experiments, the mAb was rat anti–mouse leukocyte common antigen (7) (clone M 9/1, TIB 122 from ATCC). In our initial work, the mAb were to MHC class II products (8, 9) (clones B21-2, rat anti–mouse I-A$^{b,d}$, TIB 229; and clone 14-4-5S, mouse anti–mouse I-A$^{d,k}$, HB32 [ATCC]). mAbs were added as hybridoma culture supernatant at 30% vol/vol to cells at $10^6$ cells/ml for 30 min at 4°C in RPMI-1640 culture medium supplemented with 10% FCS. The suspension was washed twice in this medium and applied to bacteriologic Petri dishes (35 or 60 mm; Falcon 1007 or 1008; Falcon Labware, Oxnard, CA) that had been coated for 1 h with a goat anti–mouse Ig in PBS (0211-0121, 32 $\mu$g per 60-mm dish; Cappel Laboratories, Cochranville, PA) or goat anti–rat Ig (01-16-12, 12 $\mu$g per 60-mm dish; Kirkegaard and Perry Laboratories, Gaithersburg, MD). The cells settled for 30 min at 4°C, and then 10 min at room temperature. Nonattached cells were removed with a Pasteur pipette after swirls of the dish and monitoring for loose cells under an inverted microscope. The attached, LC-enriched cells were then eluted by competition with either rat or mouse Ig (500 $\mu$g/ml PBS; Jackson ImmunoResearch) for 45 min at 4°C, and then pipetted into 1.5-ml conical polypropylene tubes. After centrifugation, the cells were resuspended to $3-5 \times 10^5$ cells/ml for further culture or analysis. The yields were $1-2 \times 10^6$ epidermal cells per ear and $1-3 \times 10^4$ LC/mouse. >90% of the Ia$^+$ cells that were applied to the anti-Ig plates were recovered in the adherent fraction. The same panning procedure was also used on epidermal suspensions that had been cultured 1-3 d as described (1). Before panning, the cultured suspension was treated with 0.1% trypsin to remove dead cells, and then panned with antileukocyte or anti-Ia mAb as above.

## Frequency of LC

LC enrichment and yields were determined by immunofluorescent staining with anti-Ia antibodies. We used FITC-B21-2 (anti-I-A$^{b,d}$ at 1-3 $\mu$g/ml), or biotin 14-4-5S (anti-I-E$^{d,k}$ at 3-6 $\mu$g/ml) followed by phycoerythrin avidin (Becton-Dickinson Immunocytometry Systems, Mountain View, CA). Negative controls were LC from the inappropriate haplotype and FITC–anti-Thy-1 (a rat IgG2b, isotype-matched with B21-2 and purchased from Becton-Dickinson Immunocytometry Systems). The immunofluorescent staining was performed on cells that were cytospun (Shandon Southern Instruments, Inc., Selwickley, PA) onto glass slides, or attached to poly-L-lysine–coated multiwell dishes. Alternatively, the suspensions were monitored by cytofluorography using a FACS 440 as described (3).

## Culture of Enriched LC

LC were enriched by panning to ~80% purity (see Results) and resuspended in culture medium that consisted of RPMI-1640 supplemented with 10% FCS, $5 \times 10^{-5}$ M 2-ME, 20 $\mu$g/ml gentamicin, 100 U/ml penicillin, and 25 $\mu$g/ml streptomycin. A variety of conditioned media and defined cytokines (see below) were added to look for effects on LC viability and function. $3.0-5.0 \times 10^4$ enriched LC in 0.2 ml were plated in 6-mm flat-bottom microtest plates (3596; Costar, Cambridge, MA). After overnight culture, most

of the contaminating keratinocytes had adhered to the dishes. The nonadherent population with >95% LC was removed by pipetting and cultured an additional 0–3 d. Before testing accessory function, these cultured LC were washed free of most of the culture medium by centrifuging the cells in the microculture plates. We spun the plates twice, each time at 1,000 rpm for 2 min in a Sorvall RT6000 centrifuge followed by resuspension in fresh medium. The cells were finally resuspended to 0.2 ml for viable cell counts and for use as accessory cells in stimulating the primary mixed leukocyte reaction (MLR).

## Conditioned Media

*Keratinocyte-conditioned Medium (KCM).*   Epidermal cell suspensions were plated in 100-mm plastic dishes (3003; Falcon Labware) at 15–20 × 10⁶ cells/plate in culture medium (see above). After overnight culture, few if any LC but most of the keratinocytes had adhered forming a dense but subconfluent monolayer (1). Nonadherent cells were removed by pipetting over the monolayer. The adherent cells were cultured for 2–3 d to provide KCM. Three different batches of KCM were prepared in this way and found to be active in supporting the maturation of enriched LC in culture (see Results). The medium was centrifuged at 280 $g$, filtered through 0–45-$\mu$m Millipore filters, aliquoted, and stored frozen at −20°C.

*Macrophage-conditioned Medium.*   24-h-conditioned media were prepared from the J774 and RAW mouse macrophage cell lines, thioglycollate-elicited peritoneal macrophages, and from Fc receptor–positive (macrophage-enriched) fractions of spleen adherent cells (10, 11). The cells were cultured at 5–10 × 10⁵ cells/ml for 24 h with or without stimulation with 10 $\mu$g/ml LPS (*Salmonella typhosa*; Difco Laboratories, Detroit, MI), 1 U/ml murine rIFN-$\gamma$ (Genentech Inc., South San Francisco, CA), or 50–100 U/ml murine rIL-1$\alpha$ (Hoffman-LaRoche, Inc., Nutley, NJ).

*Natural Sources of CSFs.*   The WEHI and L cell lines were used to provide conditioned media with mouse IL-3 (12) and M-CSF respectively (13). Both media actively supported the development of macrophages in liquid cultures of 0.5 × 10⁶ mouse bone marrow cells in 16-mm plastic wells in DMEM with 10% FCS and antibiotics. Purified natural mouse M-CSF (13) was kindly provided by Dr. R. Stanley (Albert Einstein Medical College, Bronx, NY).

*MLR-conditioned Medium.*   Primary MLRs were generated between 3 × 10⁴ dendritic cells and 3 × 10⁶ allogeneic T lymphocytes in 1-ml macrocultures (14). The conditioned medium was collected at 3 d.

## Purified Cytokines

We were generously provided with a large panel of recombinant cytokines (13, 15–19) by several colleagues (Table I). The cytokines with CSF activity (IL-3, GM-CSF, G-CSF, M-CSF) were tested on mouse marrow cultures (see above) to ensure their efficacy.

## Anticytokine Antibodies

Three high-titer polyclonal antisera were tested for neutralization of keratinocyte conditioned media. Rabbit anti–mouse GM-CSF serum (16) was kindly provided by Dr. S. Gillis, Immunex Corp. A 1% vol/vol dose fully neutralized 10 ng/ml rGM-CSF. Ig fractions of rabbit anti–mouse rTNF and sheep anti–mouse natural IFN-$\alpha/\beta$ were gifts of Dr. E. A. Havell, Trudeau Institute and were used at a dose of 1–5 × 10⁴ neutralizing U/ml. The antibodies were mixed with conditioned media for 1 h before addition to the LC cultures.

## Accessory Function

Accessory function was tested in the primary MLR. It was shown previously that the capacity of primary populations of leukocytes to act as MLR stimulators required some accessory function in addition to the expression of MHC-encoded transplantation antigens. For example, Ia⁺ lymphoid dendritic cells and cultured epidermal LC were powerful MLR stimulators (1, 4), while freshly isolated LC and IFN-$\gamma$-stimulated macrophages were weak in spite of the expression of large amounts of class II MHC products (1, 4, 8, 14).

Therefore stimulatory activity in the primary MLR was used as a measure of the accessory function that is required for the activation of antigen-specific, resting T cells. We added graded doses of LC, or splenic dendritic cells (10) to a fixed dose of $3 \times 10^5$ allogeneic, Ia$^-$, nylon wool–nonadherent, spleen or lymph node T cells in 0.2-ml culture medium in flat microtest wells. The LC were used with or without treatment with 900–1,500 rad of ionizing $^{137}$Cs irradiation, with similar results. On day 4 or 5 of the MLR, 1 $\mu$Ci [$^3$H]TdR was added, and [$^3$H]TdR uptake was measured 8 h later. Standard deviations of triplicate cultures were <10%, and were not shown.

## Results

*Enrichment of LC by Panning.* Ia$^+$ LC compose ~1% of ear epidermal suspensions (1), so that an enrichment method had to be developed to study the properties of LC in the absence of keratinocytes. A panning technique provided LC in sufficient purity. The bulk of the epidermal keratinocytes were killed with anti-Thy-1 and complement, and then the LC were positively selected by exposure to specific mAb and panning on anti-Ig-coated plates. In the first experiments we used anti-Ia, and in subsequent experiments, anti–leukocyte common antigen. Neither mAb bound significantly to keratinocytes (20).

By phase-contrast and immunofluorescence microscopy (Fig. 1, *A* and *B*), it was evident that panning enriched the LC from a starting incidence of ~1% to a level of 80–85%. A small subpopulation of LC stained more brightly with anti-Ia (*arrows*, Fig. 1, *B* and *C*). Quantitation by flow cytometry revealed that the mean level of LC Ia approached that of the Ia-rich dendritic cells from spleen (Fig. 1 *C*). Staining with the mAb FITC-B21-2, which specifically stains I-A$^d$ and not I-A$^k$ LC (Fig. 1 *C*), was used to monitor LC content in subsequent experiments.

To test the effect of the LC enrichment procedure on accessory function, the MLR-stimulating activity of splenic dendritic cells and cultured LC was evaluated with or without treatment with anti-Thy-1 and complement followed by panning. In eight experiments, panning shifted the dose-response curve of MLR-stimulating function by a factor of two at most, and sometimes not at all (Fig. 2, *A* and *B*, respectively).

When fresh LC were tested as MLR stimulators and compared with splenic dendritic cells (Fig. 2 *C*) or to cultured LC (Fig. 2 *D*), the day 0 LC were 10–20 times less active, which is in accordance with previous results in which LC were enriched by another method (4). We conclude that LC can be significantly enriched by panning, and that the use of this method allows one to observe the marked increase in accessory function that occurs during bulk epidermal culture.

*LC Viability and Function Is Mediated by KCM.* LC were isolated from fresh epidermal suspensions and cultured with or without supplementation with KCM. During the first day of culture, many of the contaminating keratinocytes adhered to the surface of the culture dish, and then the nonadherent, highly enriched LC could be transferred to fresh wells. LC morphology, expression of Ia, and viability (Fig. 3 *A*) were not measurably changed by the addition of KCM during the first day of culture. The expression of Ia increased fivefold with or without KCM (not shown), as noted before in bulk epidermal cultures (3). However, the addition of KCM was critical for viability on the second and third days of culture. In the absence of KCM, viable cell yields fell markedly, while with KCM, viable
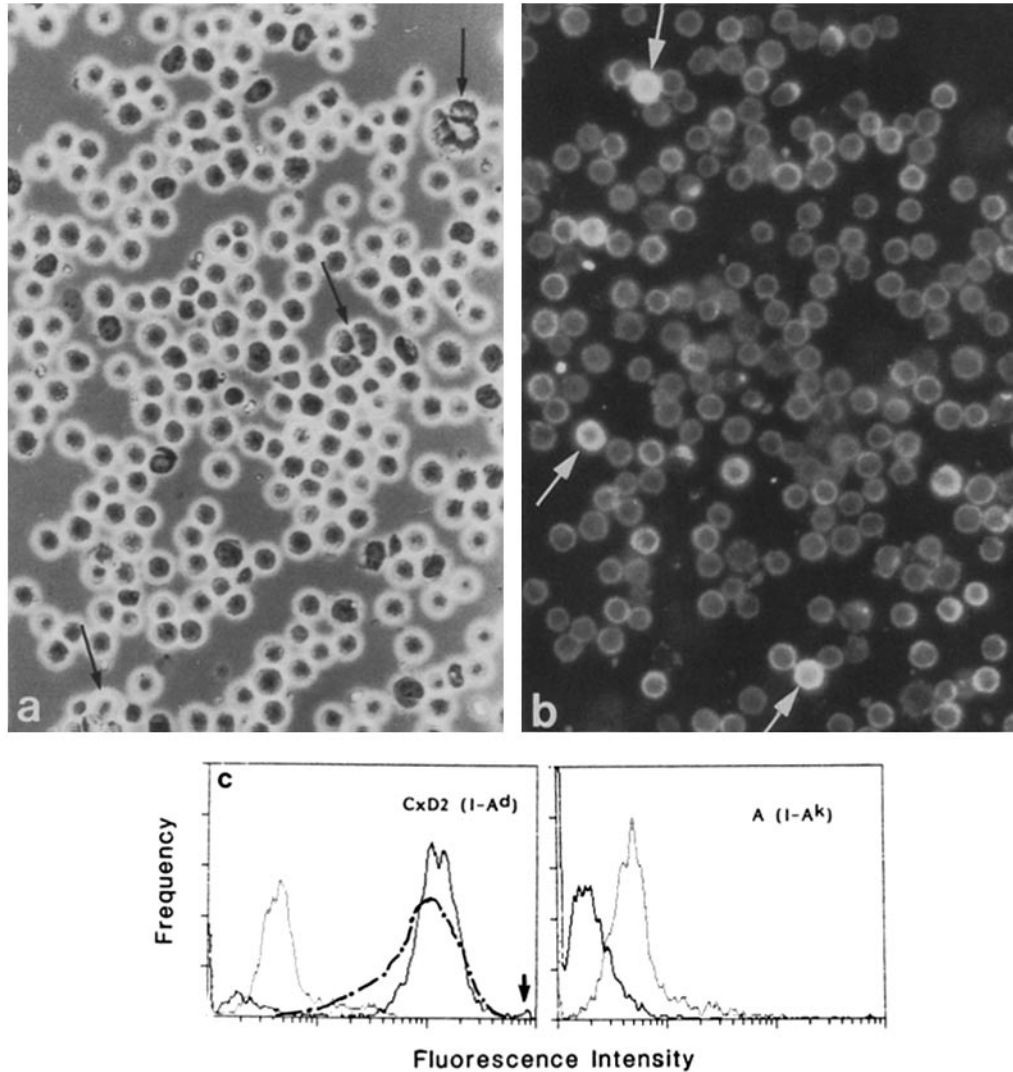
FIGURE 1.   Features of LC after enrichment by panning. *A*. Phase-contrast microscopy following adherence to poly-L-lysine–coated multiwell slides. Most of the cells have the appearance of leukocytes. The small numbers of keratinocytes (*black arrows*) are larger, have a smooth surface, and often contain cytoplasmic granules. × 300. *B*. Immunofluorescence with anti-Ia. Same field as *A*, but stained with FITC-B21-2, anti-I-A$^{b,d}$. Note that some of the LC (*white arrows*) stain more brightly. This fraction typically is <5% of the total Ia$^+$ cells. *C*. Cytofluorography. LC were panned from (C × D2)F$_1$ (I-A$^d$) and A (I-A$^k$) mice with antileukocyte mAb and compared with (C × D2)F$_1$ spleen dendritic cells (*DC*) (————). The staining reagents were FITC-B21-2, anti-I-A$^{b,d}$ (*thick line*) or the isotype-matched, IgG2b FITC-Thy-1.2 as control (*dotted line*). Thy-1 is expressed on contaminating keratinocytes but not LC (20). Note that FITC-B21-2 stains I-A$^d$ but not I-A$^k$ LC, and that the mean level of staining is comparable to splenic DC. The small subset of Ia-rich LC is evident (*arrow*).
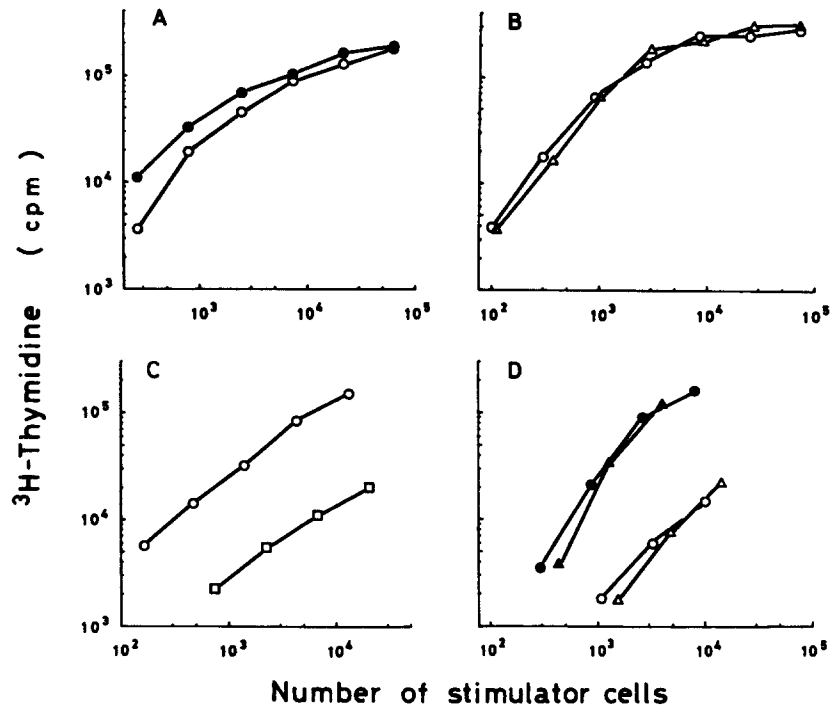
FIGURE 2. MLR-stimulating activity of panned LC and dendritic cells. Graded doses of LC or dendritic cells were added to $3 \times 10^5$ allogeneic, nylon-nonadherent, Ia$^-$, spleen cells. The MLR was monitored on day 4 by measuring [$^3$H]TdR uptake. A. Panning has little effect on the function of splenic dendritic cells. Low-density spleen adherent cells (10), from (B6 × D2)F$_1$ mice, were cultured overnight and rosetted with antibody-coated red cells to deplete macrophages. One portion of the enriched dendritic cells was panned with anti-Ia (O), and the other was not (●). The dendritic cells were then used as stimulators for the MLR of B6.H-2k T cells. B. Panning does not affect the accessory function of cultured LC. Epidermal cell suspensions were cultured 3 d. A low-density, LC-enriched fraction was isolated (50% Ia$^+$ LC) as described (1). One part of the cells was tested as MLR stimulators without further treatment (O), while the other part was enriched with anti-Thy-1 plus complement, followed by panning (Δ). C. Comparison of freshly isolated LC and splenic DC as MLR stimulators. LC were enriched by panning (□). Splenic DC (O) were low-density spleen adherent cells that were dislodged from the culture surface after 5 h of culture. D. Comparison of freshly isolated and 3-d-cultured LC as MLR stimulators. LC were enriched by panning from fresh (*open symbols*) or 3-d-cultured (*closed symbols*) epidermal suspensions. In one experiment (*circles*), the stimulators were strain A and the responders strain (C × D2)F$_1$. In the other (*triangles*), the stimulators were (B6 × D2)F$_1$ and responders (C × D2)F$_1$.

cell yields fell by a small extent (Fig. 3A). No mitotic figures were observed, but the morphology of the KCM-supplemented LC changed significantly. The LC enlarged and developed numerous dendritic and veiled processes (Fig. 3, B and C). The level of Ia on 1- and 3-d-cultured LC did not change (not shown).

In the presence of KCM, the MLR-stimulating activity of cultured LC rose >10-fold relative to that observed at time 0 (Fig. 4). The accessory function of purified LC cultured with KCM was similar to that seen with LC isolated from bulk 3-d epidermal cultures (Fig. 4). Three different KCM preparations were tested with similar results. A plateau in LC-stimulating activity was observed
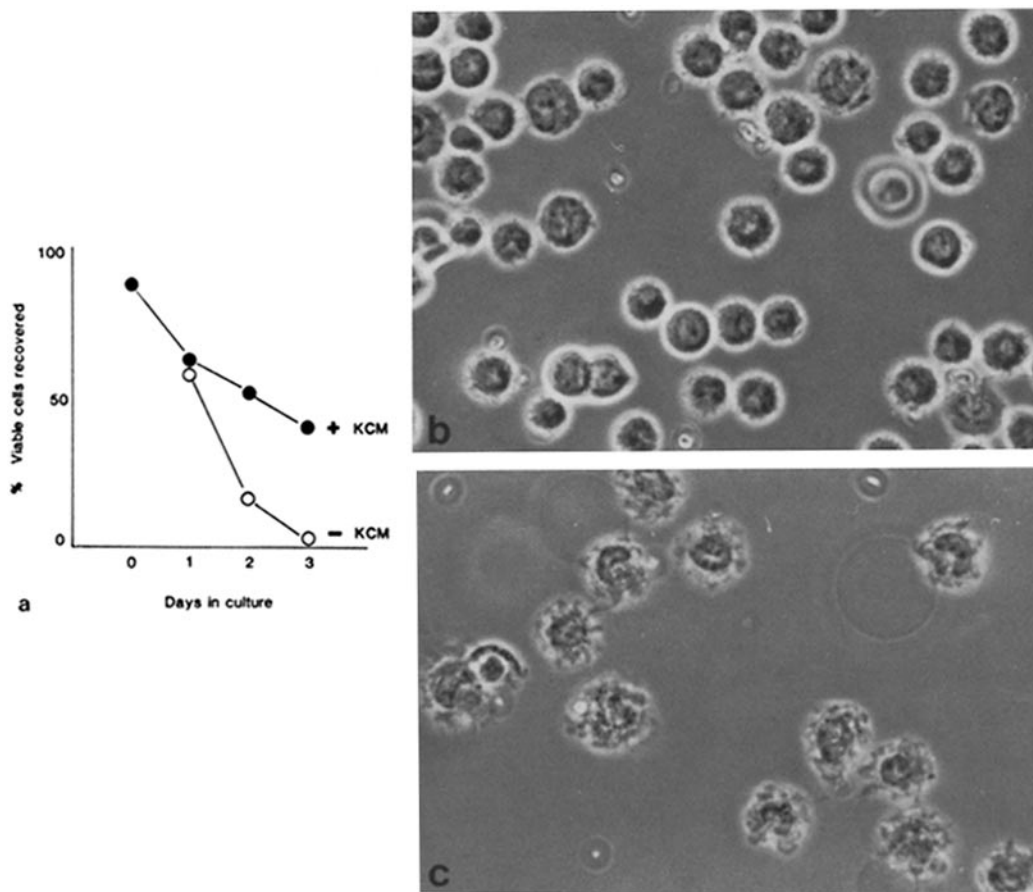
FIGURE 3.    Effect of KCM on LC viability and morphology. LC were enriched to ~80% purity by panning with anti-Ia mAb. The LC were then cultured with or without 30% (vol/vol) KCM. Most contaminating keratinocytes (20% of the starting inoculum, Fig. 1) adhered to the culture surface during the first day of culture. A. Viable cell recoveries as assessed by trypan blue staining. Data are means from three experiments. B and C. Cytologic features of 1- (B) and 3-d (C) enriched LC, cultured in the presence of 30% (vol/vol) KCM. Both populations are uniform in appearance, but the cells in 3-d cultures are larger and have longer processes. × 500.

after culture in a dose of 10–30% vol/vol KCM. We conclude that the viability and function of cultured LC is markedly enhanced in KCM.

*Effects of Other Conditioned Media on LC Viability and Function.*    The conditioned medium from LPS-stimulated macrophages was, like KCM, active in supporting the viability and function of cultured LC (Fig. 5A). Active medium was obtained from all LPS-treated macrophage populations that we tested, including the J774 and RAW cell lines, thioglycollate-elicited peritoneal macrophages, and Fc receptor–positive splenic adherent cells. LPS had no direct effect on cultured LC. Even when mixed with the medium from unstimulated macrophages, LPS did not enhance LC viability or function (not shown). rIFN-γ and rIL-1α were weak or inactive in stimulating the release of the required cytokines
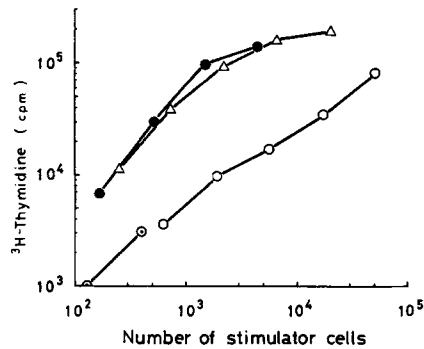
FIGURE 4. Change in LC accessory function after culture with KCM. LC were enriched from fresh epidermal suspensions by panning with anti-Ia mAb and then cultured 3 d with (●) or without (○) 75% (vol/vol) KCM. The cells were washed free of KCM and brought to a constant volume. The top stimulating dose is a reflection of relative cell yields with or without KCM. Freshly panned day 0 LC (○) were included as a comparison, as were LC isolated by panning from bulk 3-d epidermal suspension cultures (△) a part of which had been used 3 d earlier to prepare the LC that were cultured with or without KCM. In this and all subsequent experiments, stimulators were from (C × D2)F₁ mice and responders (3 × 10⁵, Ia⁻, nylon nonadherent T cells) were from strain A. [³H]TdR uptake was measured at 72–88 h.
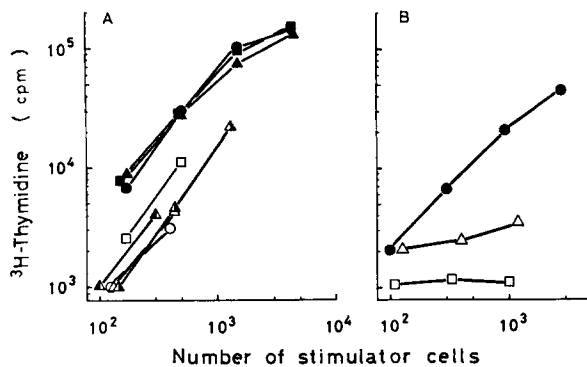


FIGURE 5. Change in LC accessory function after culture with KCM and other conditioned media. A. Macrophage-conditioned media: LC were panned from fresh epidermal suspensions and cultured for 3 d in the presence of 30% (vol/vol) macrophage-conditioned medium obtained as follows. 5 × 10⁶ adherent, thioglycollate-elicited peritoneal macrophages were cultured 24 h in 5 ml culture medium and 10 μg/ml LPS (▲), 1 U/ml murine rIFN-γ (▲), 50 U/ml murine IL-1 (▲), or no supplement (△). Additionally, 10⁶ Fc receptor–positive (isolated by rosetting with antibody-coated red cells) splenic adherent cells (10) were cultured 24 h with (■) or without (□) 10 μg/ml LPS in 1 ml medium. Companion LC preparations were cultured with (●) or without (○) KCM. Each preparation was washed and tested for MLR-stimulatory function. B. WEHI and L cell conditioned medium: LC were panned from fresh epidermal suspensions with antileukocyte mAb and cultured 2 d with 75% (vol/vol) keratinocyte- (●), L cell- (□), or WEHI- (△) conditioned media. The cells were washed and tested as MLR stimulators.

from macrophages (Fig. 5A). In contrast to mouse macrophages, LPS-stimulated human monocytes did not produce a factor that could act on mouse LC (not shown).

The conditioned medium from the syngeneic and allogeneic MLR also mediated the maturation of LC in culture (data not shown). However, the medium from the WEHI cell line and from L cells, which are used as standard sources of IL-3 and M-CSF, were repeatedly inactive in inducing LC maturation (Fig. 5B). These media did stimulate the expected development of macrophages in marrow cultures (not shown).

*GM-CSF Is the Factor that Mediates the Maturation of LC in Culture.* A panel of purified cytokines (Table I) was then added to LC cultures. Most were inactive (not shown) including: IL-1 (15), IL-2, IL-3 (19), IL-4 (18); G-CSF (17), and M-CSF (13); cachectin/TNF, IFN-α/β/γ.

However, when rGM-CSF (Fig. 6) and purified natural GM-CSF (not shown) were evaluated, the LC behaved identically to cultures supplemented with KCM or with macrophage-conditioned medium. In the presence of low doses of GM-
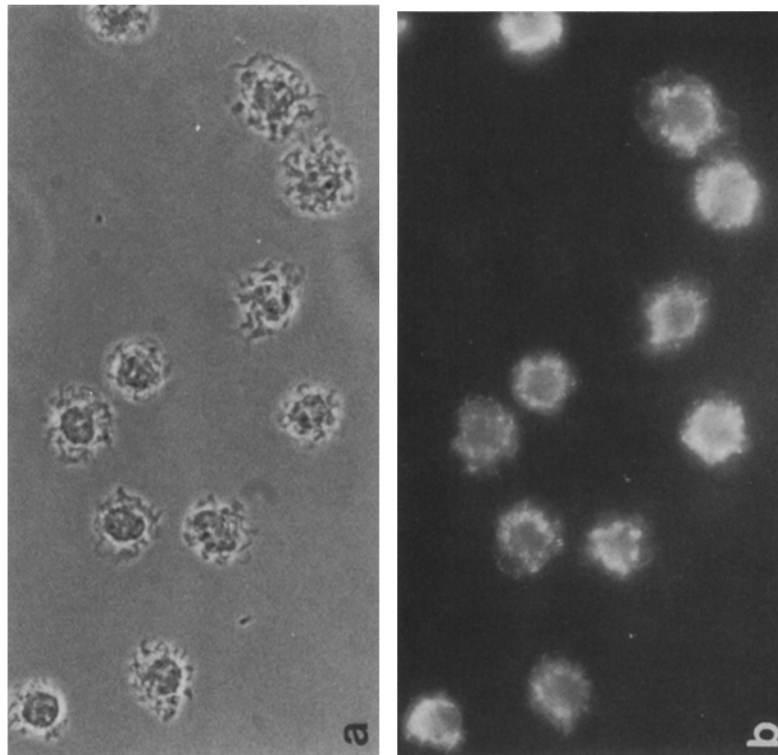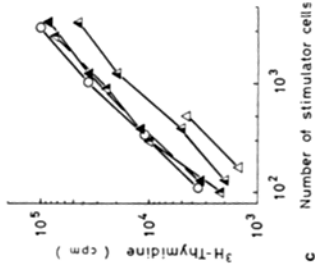
FIGURE 6.   rGM-CSF mediates the maturation of epidermal LC in culture. LC were enriched from fresh epidermal suspensions by panning with antileukocyte mAb and cultured 3 d with 1 ng/ml rGM-CSF before washing and testing for MLR-stimulating activity. A. Morphology of the cultured LC by phase-contrast, illustrating the irregular cell shape. × 500. B. Immunofluorescence of the same cells as A, using FITC–anti-Ia, B21-2. C. Comparison of 30% (vol/vol) KCM (○) with graded doses of rGM-CSF: (△) 0.01 ng/ml; (▲) 0.1 ng/ml; (▲) 1.0 ng/ml; and (▲) 10 ng/ml.

TABLE I

Cytokines Tested on Epidermal Langerhans Cells

| Cytokine | Expressed in | Activity* | Dose | Source |
|---|---|---|---|---|
| Mouse rIL-1α | E. coli | $6 \times 10^6$ | 10–20 ng/ml | P. LoMedico, Hoffman-La-Roche, Nutley, NJ |
| Mouse rIFN-γ | E. coli | $6 \times 10^6$ | 2–20 ng/ml | Genentech, South San Francisco, CA |
| Mouse rIL-3 | COS | — | 3–10% vol/vol | T. Yokota, DNAX, Palo Alto, CA |
| Mouse rIL-4 | COS | — | 200–1,000 U/ml | T. Mosmann, DNAX |
| Mouse M-CSF | Natural | $8 \times 10^7$ | 100–3,000 U/ml | R. Stanley, Albert Einstein College of Medicine, Bronx, NY |
| Mouse IFN-α/β | Natural | $10^5$ U/ml | 100–1,000 U/ml | E. Havell, Trudeau Institute, Saranac Lake, NY |
| Mouse rGM-CSF | Yeast | $>4 \times 10^7$ | 0.01–100 ng/ml | S. Gillis, Immunex, Seattle, WA |
| Mouse GM-CSF | Natural | — | 10–100 U/ml | Genzyme, Boston, MA |
| Human rIL-2 | E. coli | $5.3 \times 10^6$ | 2–20 U/ml | S. Rudnick, Biogen, Cambridge, MA |
| Human rTNF (cachectin) | Yeast | $10^8$ | 10 U/ml | B. Beutler, A. Cerami, S. Wolpe, The Rockefeller University, New York |
| Human rG-CSF | E. coli | $8 \times 10^7$ | 60 U/ml | M. Moore, Sloan Kettering, New York |

* U/mg except where stated.
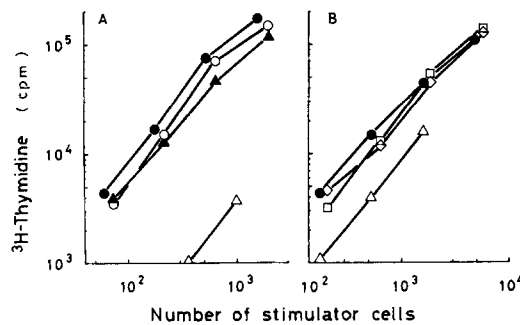


FIGURE 7. Anti-GM-CSF reduces the activity of KCM. LC were enriched from fresh epidermal suspensions by panning with antileukocyte mAb and cultured 3 d with KCM and the indicated supplements before washing and testing as MLR stimulators. A. LC were cultured with: 75% (vol/vol) KCM (●), KCM plus 1% rabbit preimmune serum (○), KCM plus rabbit anti-GM-CSF (△), or with KCM plus anti-GM-CSF plus 100 ng/ml rGM-CSF (▲). B. LC were cultured with 30% (vol/vol) KCM (●), with KCM and 1% rabbit anti-GM-CSF (△), KCM and rabbit anti-mouse TNF (□) or KCM and sheep anti-mouse IFN (◇).
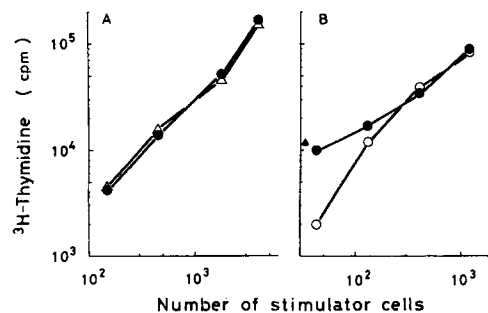


FIGURE 8. GM-CSF does not influence accessory function during the MLR. A. Enriched LC were cultured for 3 d with 10 ng/ml rGM-CSF, washed, and tested as MLR stimulators in the presence of 1% (vol/vol) rabbit anti-GM-CSF (△) or control serum (●). B. Enriched LC were cultured for 3 d with 30% KCM, washed, and then tested as MLR stimulators in the absence (○) or presence of 10 ng/ml rGM-CSF (●). The latter induced background proliferative activity, including the T cell only wells (▲), which was due to the induction of colonies of large round myeloid cells.

CSF, the LC remained viable, developed many processes, stained brightly with anti-Ia mAb, and become very active MLR stimulators (Fig. 6, A-C). Half-maximal activity was observed at ~5 pM (0.1 ng/ml; Fig. 6C).

Three polyclonal antisera were then evaluated to test if the function of KCM could be inhibited. Rabbit anti-GM-CSF blocked the effect of KCM, and the block was reversed by adding back an excess (100 ng/ml) of rGM-CSF (Fig. 7A). Rabbit anti-TNF and sheep anti-IFN-$\alpha/\beta$ did not block the effect of KCM (Fig. 7B). Taken together, the results indicate that GM-CSF is the principal if not exclusive mediator of LC maturation in culture.

*Effects of GM-CSF on the MLR.*    To ensure that the observed effects of GM-CSF were on the maturation of LC before addition to the MLR, we added GM-CSF to MLRs that were induced by graded doses of cultured LC (Fig. 8) or splenic dendritic cells (not shown). Anti-GM-CSF did not block the MLR-stimulating activity of LC that had matured in the presence of cytokine (Fig. 8A). Exogenous GM-CSF did not enhance the MLR, but it did induce myeloid colonies and some background proliferation in the unstimulated T cells (Fig. 8B). We conclude that GM-CSF mediates the immunologic maturation of LC in culture, but it does not influence subsequent dendritic or T cell function in the MLR.

## Discussion

*LC Enrichment by Panning.*    To study the mechanism whereby epidermal LC become active accessory cells during culture (1, 2, 4), it was necessary to separate this trace cell type from most of the keratinocytes in the starting epidermal suspension. We considered several enrichment methods at the outset. We were unable to remove all keratinocytes by cytotoxicity approaches (anti-Thy-1 or antidesmosomal antibodies and complement). Cytotoxicity was useful as a first step, however, and provided a preliminary enrichment from an initial level of ~1% LC to a level of 7–15% LC. Cell sorting of these partially enriched populations on the FACS could provide LC in >95% purity (4), but we encountered substantial losses in yield and viability during the sorting procedure. Therefore we coated the epidermal suspensions with either antileukocyte or anti-Ia mAb, washed, and panned on dishes coated with anti-Ig. Panning depleted >90% of the LC from the applied suspension and did not markedly reduce the accessory function of the attached cells (Fig. 2). The panned LC were sufficiently pure to establish a dependence on exogenous cytokines for viability and function (Figs. 3–8).

*The Effects of Keratinocyte-conditioned Medium and GM-CSF on Cultured LC.*    The addition of appropriate cytokines to enriched populations of fresh LC had three effects over a 2–3-d period of culture: cell viability was maintained at ~50% of the starting inoculum; the LC became larger and exhibited many processes and veils; and stimulating capacity in the primary MLR increased 10–20-fold. As a result of the combined effects on viability and function, the stimulating activity of 3-d cultured LC populations was typically enhanced 100-fold by exogenous cytokines.

The actual increase in the stimulating activity of individual LC might have been even greater than is suggested by our data (Fig. 4). This is because some of

the activity that was seen with fresh populations could be due to a fraction of Ia-rich LC (Fig. 3, B and C) that either were in the process of maturing in situ or had been exposed to small amounts of GM-CSF during the preparation of the epidermal suspension. It is possible that the majority of LC in the starting population have insignificant accessory function.

The increased accessory activity that was induced by KCM or GM-CSF might be due to three effects. One relates to improved viability (Fig. 3), which is a characteristic effect of CSFs on their target cells (21, 22). We suspect that enhanced viability is not the only effect of GM-CSF since, in isolated instances (perhaps related to higher levels of contaminating keratinocytes), LC viability was maintained close to the level of that seen with GM-CSF, but little or no increase in accessory function was observed. A second factor is the increase in the level of Ia or class II products that occurs in culture (3). Freshly isolated LC have similar amounts of Ia to splenic dendritic cells (Fig. 1), but are much less active as MLR stimulators (Fig. 2C). We have also found that Ia levels increase in cells that are cultured in the absence of KCM for a day, yet there is little increase in accessory function (M. Witmer-Pack, unpublished observations). Therefore the levels of Ia on LC do not seem to be the sole determinant of the level of accessory function. We propose that GM-CSF exerts a third differentiation effect that allows the LC to become an active accessory cell comparable to the dendritic cells isolated from lymphoid organs and lymph. This differentiation results in an accessory cell that can cluster and activate resting, antigen-specific T cells in a manner analogous to that seen with lymphoid dendritic cells (4).

*Implications of Our Data in Pinpointing the Lineage of Langerhans Cells.* Both LC and lymphoid dendritic cells are bone marrow derived (23, 24), but their precise lineage is not established. It is of interest that GM-CSF mediates differentiation of both major types of myeloid cells, granulocytes and macrophages, whereas G- and M-CSF are more lineage specific (21, 22). Because LC are influenced by GM-CSF but not by G- or M-CSF, we suggest that LC represent a type of myeloid lineage that is distinct from typical phagocytes. This would be consistent with prior data demonstrating stable differences in phenotype and function between dendritic cells and mononuclear phagocytes (25).

*Comparison of the Effects of GM-CSF on LC with the Effects of Other Cytokines on Accessory Cells.* It is becoming evident that the accessory function of dendritic cells for primary T cell responses like the MLR can be increased by cytokines. IL-1 amplifies the function of lymphoid dendritic cells about threefold, but the cells are already very active before exposure to cytokine (26). GM-CSF does not increase the function of mature LC (Fig. 8) or splenic dendritic cells (data not shown). It instead acts on freshly isolated LC, which quantitatively are weak stimulators of the MLR and polyclonal T cell mitogenesis (1, 4). GM-CSF therefore may be specialized to mobilize active dendritic cells from a weakly active, nonlymphoid tissue source.

Two other T cell–derived products enhance the expression of class II MHC products but do not appear to induce accessory cells for primary responses. IFN-γ increases the expression of Ia on mononuclear phagocytes (27) and many nonleukocytes. The lymphokine-treated macrophages do not act as stimulators of the primary MLR (8, 26), but are better presenting cells for sensitized CD4+

T lymphocytes (14). IL-4 or BSF-1 is a cytokine that enhances Ia on small mouse B lymphocytes (28). Here too the upregulated B cells are weak in stimulating a primary MLR but are active in presenting alloantigens to freshly sensitized T lymphoblasts (J. Metlay, M. Birkeland, R. Steinman, and E. Pure; unpublished results).

It is noteworthy that both cytokines that modulate the function of dendritic cells for primary responses can be released in the absence of sensitized T cells. IL-1 and GM-CSF are produced by such non-T cells as macrophages and keratinocytes. Therefore these cytokines could be released during the afferent limb of the immune response, perhaps in nonlymphoid organs where many antigens are first deposited, where they may be critical for the production of active dendritic cells. In contrast, IFN-γ and IL-4 are the products of T cells that have already begun to respond to antigen. IFN-γ and IL-4 might be expected to enhance T cell–macrophage or T–B cell interactions in the efferent limb of immunity.

We are extending our studies to test if GM-CSF alters dendritic cell function in situ. Given the role of GM-CSF as a mediator of dendritic cell maturation in culture, we are interested in testing if this cytokine can mobilize dendritic cells from tissues and/or act as an adjuvant during primary immune responses.

## Summary

A panning method has been developed to enrich Langerhans cells (LC) from murine epidermis. In standard culture media, the enriched populations progressively lose viability over a 3-d interval. When the cultures are supplemented with keratinocyte-conditioned medium, LC viability is improved and the cells increase in size and number of dendritic processes. Accessory function, as monitored by stimulating activity in the mixed lymphocyte reaction (MLR), increases at least 10–20-fold. The conditioned media of stimulated macrophages and T cells also support the viability and maturation of cultured LC. A panel of purified cytokines has been tested, and only granulocyte/macrophage colony-stimulating factor (GM-CSF) substitutes for bulk-conditioned medium. The recombinant molecule exhibits half-maximal activity at 5 pM. Without activity are: IL-1–4; IFN-$\alpha/\beta/\gamma$; cachectin/TNF; M- and G-CSF. A rabbit anti-GM-CSF specifically neutralizes the capacity of keratinocyte-conditioned medium to generate active LC. However, GM-CSF is not required for LC function during the MLR itself.

We conclude that the development of immunologically active LC in culture is mediated by GM-CSF. The observation that these dendritic cells do not respond to lineage-specific G- and M-CSFs suggests that LC represent a distinct myeloid differentiation pathway. Because GM-CSF can be made by nonimmune cells and can mediate the production of active dendritic cells, this cytokine provides a T-independent mechanism for enhancing the sensitization phase of cell-mediated immunity.

# References

1. Schuler, G., and R. M. Steinman. 1985. Murine epidermal Langerhans cells mature into potent immunostimulatory dendritic cells in vitro. *J. Exp. Med.* 161:526.

2. Schuler, G., N. Romani, and R. M. Steinman. 1985. A comparison of murine epidermal Langerhans cells with spleen dendritic cells. *J. Invest. Dermatol.* 85:99s.

3. Witmer, M. D., J. Valinsky, W. Olivier, and R. M. Steinman. 1988. Quantitation of surface antigens on cultured murine epidermal Langerhans cells: rapid and selective increase in the level of surface MHC products. *J. Invest. Dermatol.* In press.

4. Inaba, K., G. Schuler, M. D. Witmer, J. Valinsky, B. Atassi, and R. M. Steinman. 1986. The immunologic properties of purified Langerhans cells: distinct requirements for the stimulation of unprimed and sensitized T lymphocytes. *J. Exp. Med.* 164:605.

5. Wysocki, L. J., and V. L. Sato. 1978. "Panning" for lymphocytes: a method for cell selection. *Proc. Natl. Acad. Sci. USA.* 75:2844.

6. Scheid, M., E. A. Boyse, E. A. Carswell, and L. J. Old. 1972. Serologically demonstrable alloantigens of mouse epidermal cells. *J. Exp. Med.* 135:938.

7. Springer, T. A. 1980. Cell-surface differentiation in the mouse. Characterization of "jumping" and "lineage" antigens using xenogeneic rat monoclonal antibodies. *In* Monoclonal Antibodies. R. H. Kennett, T. J. McKearn, and K. B. Bechtol, editors. Plenum Press, New York. 185.

8. Steinman, R. M., N. Nogueira, M. D. Witmer, J. D. Tydings, and I. S. Mellman. 1980. Lymphokine enhances the expression and synthesis of Ia antigen on cultured mouse peritoneal macrophages. *J. Exp. Med.* 152:1248.

9. Ozato, K., N. M. Mayer, and D. H. Sachs. 1982. Monoclonal antibodies to mouse major histocompatibility complex antigens. *Transplantation (Baltimore).* 34:113.

10. Steinman, R. M., G. Kaplan, M. D. Witmer, and Z. A. Cohn. 1979. Identification of a novel cell-type in peripheral lymphoid organs of mice. V. Purification of spleen dendritic cells, new surface markers, and maintenance in vitro. *J. Exp. Med.* 149:1.

11. Nussenzweig, M. C., R. M. Steinman, J. C. Unkeless, M. D. Witmer, B. Gutchinov, and Z. A. Cohn. 1981. Studies of the cell surface of mouse dendritic cells and other leukocytes. *J. Exp. Med.* 154:168.

12. Lee, J. C., A. J. Hapel, and J. N. Ihle. 1982. Constitutive production of a unique lymphokine (IL-3) by the WEH1-3 cell line. *J. Immunol.* 128:2393.

13. Tushinski, R. J., I. T. Oliver, L. J. Gilbert, P. W. Tynan, J. R. Warner, and E. R. Stanley. 1982. Survival of mononuclear phagocytes depends on a lineage-specific growth factor that the differentiated cells selectively destroy. *Cell.* 28:71.

14. Inaba, K., and R. M. Steinman. 1984. Resting and sensitized T lymphocytes exhibit distinct stimulatory (antigen presenting cell) requirements for growth and lymphokine release. *J. Exp. Med.* 160:1717.

15. LoMedico, P. T., U. Gubler, C. P. Hellman, M. Dukovich, J. G. Giri, Y.-C. E. Pan, K. Collier, R. Semionow, A. O. Chua, and S. B. Mizel. 1984. Cloning and expression of murine interleukin-1 cDNA in *Escherichia coli. Nature (Lond.).* 312:458.

16. Mochizuki, D. Y., J. R. Eisenman, P. J. Conlon, L. S. Park, and D. L. Urdal. 1986. Development and characterization of antiserum to murine granulocyte-macrophage colony-stimulating factor. *J. Immunol.* 136:3706.

17. Souza, L. M., T. C. Boone, J. Gabrilove, P. H. Lai, K. M. Zsebo, D. C. Murdock, V. R. Chazin, J. Bruszewski, H. Lu, K. K. Chen, J. Barendt, E. Platzer, M. A. S. Moore, R. Mertelsmann, and K. Welte. 1986. Recombinant human granulocyte colony-stimulating factor: Effects on normal and leukemic myeloid cells. *Science (Wash. DC).* 232:61.

18. Lee, F., T. Yokota, T. Otsuka, P. Meyerson, D. Villaret, R. Coffman, T. Mosmann,

D. Rennick, N. Roehm, C. Smith, A. Zlotnik, and K-I. Arai. 1986. Isolation and characterization of a mouse interleukin cDNA clone that expresses B-cell stimulatory factor 1 activities and T-cell and mast-cell-stimulating activities. *Proc. Natl. Acad. Sci. USA.* 83:2061.

19. Yokota, T., F. Lee, D. Rennick, C. Hall, N. Arai, T. Mosmann, G. Nabel, H. Cantor, and K-I. Arai. 1984. Isolation and characterization of a mouse cDNA clone that expresses mast-cell growth factor activity in monkey cells. *Proc. Natl. Acad. Sci. USA.* 81:1070.

20. Romani, N., G. Stingl, E. Tschachler, M. D. Witmer, R. M. Steinman, E. M. Shevach, and G. Schuler. 1985. The thy-1 bearing cell line of murine epidermis: a leukocyte distinct from Langerhans cells and perhaps related to NK cells. *J. Exp. Med.* 161:1368.

21. Metcalf, D. 1985. The granulocyte-macrophage colony-stimulating factors. *Science (Wash. DC).* 229:16.

22. Nicola, N. A., and M. Vadas. 1984. Hemopoietic colony-stimulating factors. *Immunol. Today.* 5:76.

23. Steinman, R. M., D. S. Lustig, and Z. A. Cohn. 1974. Identification of a novel cell type in peripheral lymphoid organs of mice. III. Functional properties in vivo. *J. Exp. Med.* 139:1431.

24. Pugh, C. W., G. G. MacPerson, and H. W. Steer. 1983. Characterization of non-lymphoid cells derived from rat peripheral lymph. *J. Exp. Med.* 157:1758.

25. Steinman, R. M. 1981. Dendritic cells. *Transplantation (Baltimore).* 31:151.

26. Koide, S. L., K. Inaba, and R. M. Steinman. 1987. Interleukin-1 enhances T-dependent immune responses by amplifying the function of dendritic cells. *J. Exp. Med.* 165:515.

27. Steeg, P. S., R. N. Moore, H. M. Johnson, and J. J. Oppenheim. 1982. Regulation of murine macrophage Ia antigen expression by a lymphokine with immune interferon activity. *J. Exp. Med.* 165:1780.

28. Noelle, R., P. H. Krammer, J. Ohara, J. W. Uhr, and E. S. Vitetta. 1984. Increased expression of Ia antigens on resting B cells: An additional role for B-cell growth factor. *Proc. Natl. Acad. Sci. USA.* 81:6149.