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# IMMUNOLOGIC PROPERTIES OF PURIFIED EPIDERMAL LANGERHANS CELLS

## Distinct Requirements for Stimulation of Unprimed and Sensitized T Lymphocytes

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A critical requirement for the initiation of cell-mediated immune responses *in vitro* and *in vivo* is the administration of antigen in association with a subset of leukocytes termed dendritic cells (reviewed in 1). Shortly after sensitization, the primed T cell can interact with antigen in association with other antigen-presenting cells. For the most part, prior studies have used dendritic cells from lymphoid organs, afferent lymph, and blood. Recent data (2–4) indicate that dendritic cells are also present in nonlymphoid tissues. A particularly large pool are the dendritic or Langerhans cells (LC)<sup>1</sup> of the epidermis (4). In the mouse, the number of LC is some 20 times greater than the number of splenic dendritic cells. However, epidermal cell suspensions are relatively weak accessory cells when freshly isolated, and only become active stimulators of T cell proliferation after 2–3 d of culture (4).

To clarify the immunologic capacities of tissue dendritic cells, we have purified LC after 12 or 72 h in culture. We have found that 12-h-cultured LC actively present antigens to primed lymphocytes, but they are weak or inactive in sensitizing T cells as in the mixed leukocyte reaction (MLR) and T-dependent antibody formation. In contrast, 72-h LC are the most active stimulators of primary responses yet to be identified. These data suggest that the maturation of tissue dendritic cells represents a new control point in the stimulation of immunity. This control is distinct from antigen presentation *per se*, and seems to involve the capacity of dendritic cells to bind to T cells by an antigen-independent pathway.

### Materials and Methods

*Purification of LC.* Epidermal cell (EC) suspensions were prepared from ears of BALB/c, (C × D2)F<sub>1</sub>, and B10.A(3R) mice (4). LC, which represented ~2% of total EC, were selectively stained with FITC-labeled B21-2 anti-Ia mAb, since LC are the principal if not exclusive EC that expresses Ia in the steady state (5). Sorting was performed on FACS II and IV instruments (Becton Dickinson Immunocytometry Systems, Mountain View, CA) at 1,000 cells/s. The fluorescent fraction was >95% pure and the Ia<sup>-</sup> fraction lacked detectable LC (see Results). Before sorting, the LC were partially enriched on the basis

<sup>1</sup> *Abbreviations used in this paper:* EC, epidermal cells; LC, Langerhans cells.

of their nonadherence to plastic and low buoyant density in albumin columns (4). After 12 h of culture, the nonadherent low-density EC were 7–17% Ia<sup>+</sup>, and after 72 h, 30–60% were Ia<sup>+</sup>.

*Immune Responses in Culture.* Three T-dependent responses were monitored as specified in the Results: the primary and secondary MLR (6, 7), the primary antibody response to SRBC and TNP-KLH (8, 9), and the secondary response of in vitro-primed cells to KLH (9). In each case, we tested 12- and 60-h LC as well as 12-h splenic dendritic cells (10) as accessory cells. The responding T cells were LYt-2<sup>-</sup> unprimed lymphocytes, or primed lymphoblasts or memory cells prepared as described (6–9). The primed populations were >50% antigen specific, as monitored with accessory T cell binding assays (11).

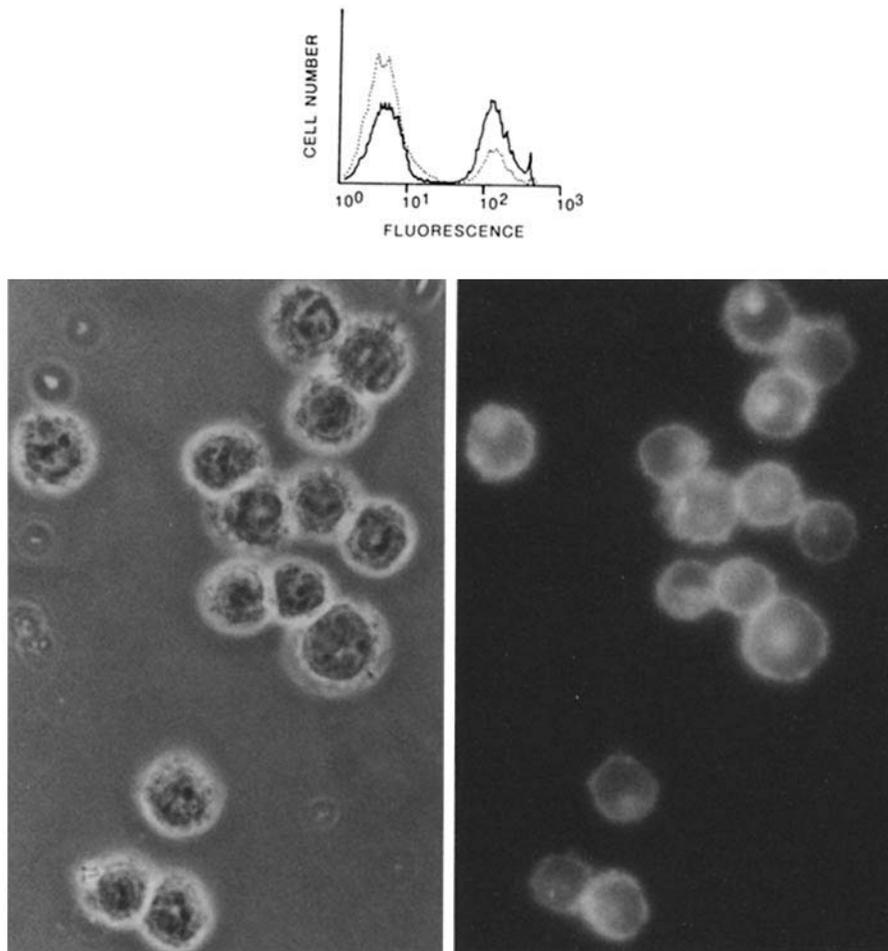
*Accessory Cell-T Cell Binding Assays.* T lymphoblasts that had been sensitized to alloantigen, lectin, or KLH were tested for their capacity to bind to LC or spleen dendritic cells as described (11). Briefly, the T cells were labeled with carboxyfluorescein diacetate, sedimented with graded doses of accessory cells, and allowed to form aggregates in the presence or absence of antigen at 4 or 37°C. After 10–20 min, the cells were gently resuspended and the extent of T cell clustering was monitored by counting single nonfluorescent cells.

## Results

EC suspensions were cultured for 12 and 72 h before study of the trace Ia<sup>+</sup> LC component. Earlier work (4) had shown that EC acquire enhanced accessory cell function during 3 d of culture, and that the Ia<sup>-</sup> (predominantly keratinocytes) component was not immunosuppressive at the doses used. 12 h was chosen as the first time point for two reasons. The amount of Ia on isolated LC increases rapidly, rising to a plateau in the first 12 h of culture (M. Witmer, J. Valinsky, W. Olivier, R. Steinman, in preparation). Also by 12 h, most LC had acquired a low buoyant density (4), which facilitated their purification. The capacity of 12-h (fresh) and 60–72-h (cultured) LC to stimulate unprimed and sensitized T cells was evaluated in several assays.

*Isolation of LC by Cell Sorting.* To properly study the immunologic properties of LC, it was necessary to purify these cells away from most Ia<sup>-</sup> EC, since the latter could enhance LC function and/or development. The LC enrichment that could be obtained by negative selection (killing with anti-Thy-1 and antidesmosomal antibodies plus complement) never exceeded 60%. We therefore turned to the much slower procedure of cell sorting, which has been used previously (12) to purify murine LC. When we stained suspensions with FITC-labeled anti-Ia, 12-h and 60–72-h LC had identical amounts of Ia (Fig. 1, *top*). Of the many antibodies that selectively stained LC (5), only anti-Ia stained strongly enough to provide a clear cut separation from keratinocytes. <3% of the cells had to be discarded during the sorting runs. The nonfluorescent fraction lacked detectable Ia<sup>+</sup> cells, while the fluorescent fraction was 95–99% pure (Fig. 1, *bottom*). Under a phase-contrast microscope, all the Ia<sup>+</sup> cells were covered with short, fine processes. The nucleus was irregular in shape and occupied roughly one-half of the total cell profile. The few contaminating keratinocytes that were observed varied considerably in size, had distinctive granules, and lacked dendritic processes.

*12-h LC Stimulate Primed, But Not Unprimed, T Cells.* While several studies have concluded that LC are potent stimulator cells for the primary MLR (reviewed in 13), dose-response curves with sorted 12-h LC indicated otherwise (Fig. 2, *middle and right*). In contrast, 60-h LC were as active as spleen dendritic



**FIGURE 1.** *Top:* Expression of Ia on 12- and 60-h EC suspensions. Nonadherent EC were collected after 12 (dotted line) or 60 h (solid line) of culture, floated in dense albumin columns (4), and stained with a saturating dose of FITC-labeled anti-Ia<sup>b,d</sup> mAb (clone B21-2). A well-delineated Ia<sup>+</sup> subset is seen in both 12- and 60-h EC, and accounts for 17 and 49% of the total EC, respectively. Similar results were obtained in two other experiments. Not shown are controls in which the left peak of weak fluorescence was shown to be background, as monitored by application of FITC-B21-2 to B6.H-2k EC. Note that the amount of Ia on 12- and 60-h EC do not differ, and that the distribution of Ia is uniform for each population. The small peak at the far right represents aggregates of fluorescent LC. Aliquots of the same cell suspensions were fixed in 3% formaldehyde in PBS for 30 min at room temperature, washed and exposed to 1  $\mu$ g/ml (saturating) <sup>125</sup>I-B21-2 in suspension. The average level of bound B21-2 was  $6 \times 10^5$  molecules/cell, which was two to three times the level of Ia on spleen dendritic cells (not shown, but see ref. 4). *Bottom:* The appearance of sorted 12-h Ia<sup>+</sup> LC. Ia<sup>+</sup> EC were allowed to attach to poly-L-lysine-coated coverslips at 4°C and fixed in 3% formaldehyde before examination by phase-contrast (*left*) or immunofluorescence microscopy (*right*). See text for details. 750  $\times$ .

cells and 100–300 times more active than 12-h LC (Fig. 2, *right*). Sorted 12-h LC were weaker stimulators than unseparated anti-Ia-coated EC (Fig. 2, compare  $\Delta$  with  $\blacktriangle$  in *middle* and *right*), suggesting that the presence of Ia<sup>-</sup> keratinocytes

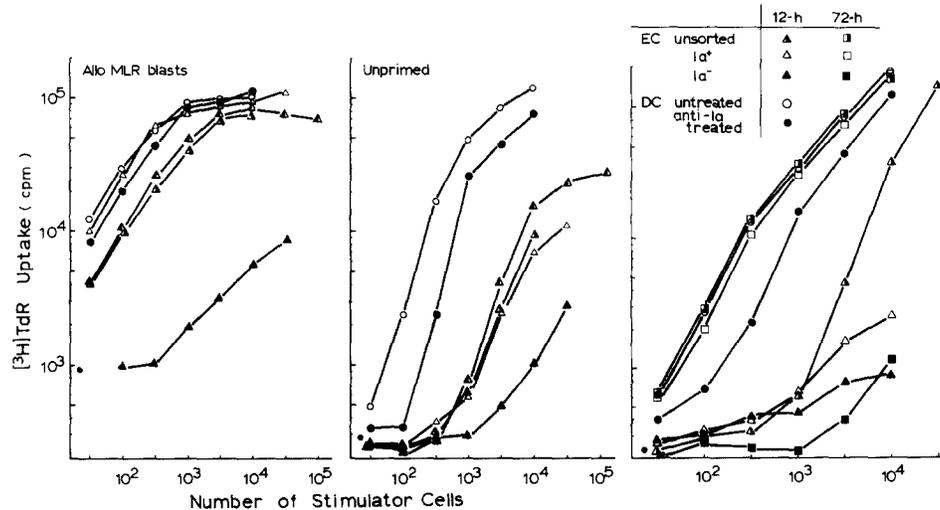


FIGURE 2. Freshly isolated Ia<sup>+</sup> LC do not stimulate the primary MLR, but can stimulate alloreactive T blasts. The Lyt-2<sup>-</sup> T cells were from B10.A (3R) mice, and were either unprimed or primed to (C × D<sub>2</sub>)F<sub>1</sub> allogeneic DC. The APC were obtained from (C × D<sub>2</sub>)F<sub>1</sub> mice, and included: unsorted EC that were coated with FITC-anti-Ia; Ia<sup>+</sup> and Ia<sup>-</sup> EC fractions from the cell sorter; and spleen dendritic cells that were or were not coated with FITC-anti-Ia. For the latter unsorted cells, the abscissa is the actual number of cells (10 and 50% Ia<sup>+</sup> for 12- and 60-h LC, respectively). The T cell doses are 3 × 10<sup>5</sup> for unprimed (*middle* and *right*), and 2 × 10<sup>4</sup> for primed blasts (*left*). [3H]TdR uptakes were measured at 72–84 h for unprimed T cells, and at 20–26 h for T blasts. Note that the 12-h Ia<sup>+</sup> LC (Δ) are as active as anti-Ia-coated DC (●) in the stimulation of T blasts (*left*), but are ~3% as active in stimulating unprimed T cells in both experiments (*middle* and *right*). 72-h Ia<sup>+</sup> LC (□; *right*) are threefold more active than anti-Ia-coated DC, and >100 times more active than 12-h Ia<sup>+</sup> LC.

was indeed allowing some degree of LC maturation during the course of the MLR assay. We were concerned that the use of anti-Ia antibody to sort the LC would inhibit their function. Some inhibition was noted when spleen dendritic cells were coated with FITC-B21-2 in the primary MLR, but the stimulatory capacity was still readily detectable (Fig. 2).

In contrast to the primary MLR, 12-h LC exhibited strong stimulatory capacity for alloreactive T blasts (Fig. 2, *left*). The sorted LC were, on average (four experiments), as active as spleen dendritic cells.

The experiments were extended using primary and secondary responses to the protein, KLH. For primary responses, we measured antibody formation to TNP-KLH, as this requires the sensitization of KLH-specific helper T cells *in vitro*. For secondary responses, we measured the proliferation of T blasts that had been primed to KLH (9). Consistent with the MLR results, 12-h LC did not act as stimulators for antibody responses to TNP-KLH or to SRBC (Fig. 3). 60-h LC and spleen dendritic cells were extremely active, and gave close to maximal responses at accessory cell/lymphocyte ratios of 1:300. In contrast to the primary response, 12-h LC could present KLH to primed, Lyt-2<sup>-</sup> helper T blasts, and were comparable to spleen dendritic cells (Fig. 4). We conclude that highly enriched, 12-h LC can stimulate primed but not unprimed T cells.

*LC/T Lymphocyte Binding Assays.* In the primary MLR and T-dependent

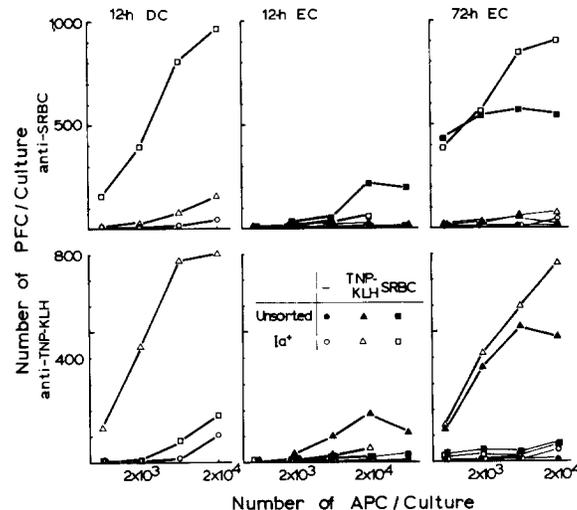


FIGURE 3. Cultured but not freshly isolated Ia<sup>+</sup> LC induce primary T-dependent antibody responses. Unsorted and Ia<sup>+</sup> EC were prepared from 12- and 60-h EC cultures, and compared to spleen DC as accessory cells for the antibody response to two thymus-dependent antigens; SRBC and TNP-KLH, or no antigen. The responders were mixtures of  $5 \times 10^6$  B and T lymphocytes (Sephadex G10-nonadherent spleen cells). Ia<sup>-</sup> EC were totally inactive, and the data are not shown. Direct PFC were measured at 4 d, and the APC were exposed to 900 rad <sup>137</sup>Cs. PFC were measured on indicator SRBC (*top*) or TNP-modified SRBC (*bottom*). Note that 12-h dendritic cells and 60-h LC are potent accessory cells, and support responses that are antigen-dependent and specific. Sorted 12-h LC are inactive. Some function is noted in unsorted 12-h EC, presumably because some LC mature during the course of the antibody response (4 d).

antibody response, the responding lymphocytes aggregate with the stimulating dendritic cells (6, 8). In the course of the above studies, it was apparent that fresh LC did not initiate the formation of cell aggregates, in spite of their ability to present antigen in some assays. Cultured LC and spleen dendritic cells did induce cluster formation. It is also known (11) that T cells can bind to APC by separate antigen-dependent and -independent pathways. The former occurs with most types of Ia<sup>+</sup> accessory cells, and at 4°C or 37°C; the latter occurs with dendritic cells, but only at 37°C. Therefore, we sensitized T blasts to alloantigens, lectin, or to KLH, and we assessed the LC/T cell interaction in binding assays. A particular interesting feature of the LC system is that 12-h and 60–72-h LC express similar amounts of Ia (Fig. 1). We verified that the clustering capacity of spleen dendritic cells was not altered after exposure to B21-2 anti-Ia (not shown).

The binding assays showed that 12-h and 60-h LC could cluster primed T cells in an antigen-dependent fashion, and that LC were as active as spleen dendritic cells (Fig. 5, *right* and *left*). In the experiment shown in Fig. 5, *left*, clustering with KLH-primed blasts required addition of KLH. In other experiments, clustering to alloreactive blasts did not occur if the LC and T cells were syngeneic, as described previously (11). However, 12-h LC exhibited little antigen-independent binding to lectin-induced blasts at 37°C (Fig. 5, *middle*), whereas 60-h LC and spleen dendritic cells were active. These binding assays directly confirm that freshly isolated LC present antigen to T cells but lack the ability to cluster T cells by the antigen-independent pathway.

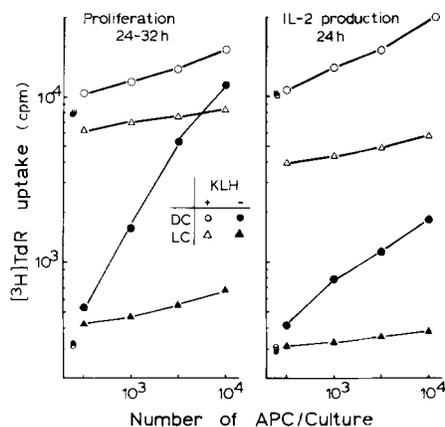


FIGURE 4. Freshly isolated Ia<sup>+</sup> LC stimulate the growth of KLH-primed T blasts. Lyt-2<sup>-</sup> T cells were primed to KLH (8) and rechallenged with or without 50 μg/ml KLH using 12-h spleen dendritic cells or sorted 12-h LC as presenting cells. The spleen dendritic cells were not coated with B21-2, and this probably accounts for the syngeneic MLR that occurs in the absence of KLH at relatively high cell doses. Proliferation (*left*) was measured with  $3 \times 10^4$  T blasts at 24–32 h. IL-2 release (*right*) was assayed on a 50% vol/vol aliquot of the culture medium taken at 24 h. The level of IL-2 in the cultures with 300 APC was about half-maximal for the assay, and corresponded to 2 U/ml. The effect of exogenous excess human rIL-2 (10 U/ml; Biogen, Cambridge, MA) with (□) or without (■) KLH is at the top left, and backgrounds (no APC) with (○) or without (○) KLH at the bottom left.

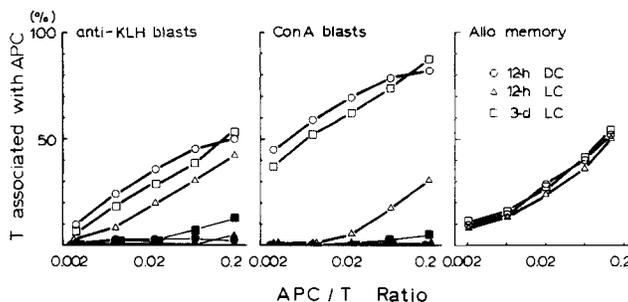


FIGURE 5. Binding of purified 12- and 60-h LC, and 12-h dendritic cells to Lyt-2<sup>-</sup> T blasts. *Left*: KLH-primed T blasts at 4°C with (open symbols) or without (closed symbols) KLH in the reaction mixture. *Middle*: Con A blasts at 37°C (open symbols) or at 4°C (closed symbols). *Right*: alloreactive (anti-H-2d) memory cells tested at 4°C, where clustering is antigen-specific (11). Note that 12-h LC present KLH and alloantigens as well as the other APC, but do not cluster in an antigen-independent fashion, here shown with Con A-induced blasts.

*Trypsin Does Not Alter Immunologic Properties of LC and Dendritic Cells.* Prior work (4) had shown that the immunostimulatory function of dendritic cells and cultured LC was not altered by trypsin. Here, we verified that trypsinization did not alter the more rapid T cell-binding functions of dendritic cells in both antigen-dependent and -independent clustering assays (data not shown). Elsewhere (M. D. Witmer, J. Valinsky, W. Olivier, R. Steinman, manuscript in preparation), we will describe how trypsinization does not alter the expression of several LC surface antigens, including class I and II MHC products.

### Discussion

An extensive literature, beginning with the work of Stingl et al. (reviewed in 13), has indicated that LC present antigens to T cells. Earlier researchers were not aware of the fact that LC mature during 3 d of culture (4), which is within the time frame of the 3–7-d assays that are used to monitor antigen presentation. This paper considers several features of LC that have been purified after 12 or 60–72 h of epidermal culture. Purification is proving to be important to the analysis of LC function, since our ongoing studies indicate that epidermal cells and their products are responsible for the immunologic maturation of LC that we have observed in culture. The yields with the current method are small ( $1\text{--}2 \times 10^4$  LC/mouse), since only small areas of ear epidermis can be handled without risking contamination with dermal cells. Nevertheless LC are an important cell type for understanding the initiation of immune responses. Whereas prior analyses (6–9) of primary and secondary responses have depended upon a comparison of dendritic cells with other leukocytes, LC represent a single cell type that acquires stimulatory function in culture.

If one restricts the use of the term “antigen presentation” to the process whereby MHC products and antigen are recognized by T cells, then purified LC are active presenting cells when studied shortly (12 h) after epidermal dissociation. 12-h LC express very high levels of the class II MHC products that are required during antigen recognition by most helper cells (Fig. 1), and 12-h LC actively bind and stimulate sensitized Lyt-2<sup>-</sup> T cells in an antigen-dependent fashion (Figs. 2, 4, and 5). Our dose-response studies indicate that 12-h LC interact with primed T cells in a manner that is comparable to spleen dendritic cells. These same LC populations, however, cannot sensitize T cells, as monitored by the MLR or T-dependent antibody responses. 60–72-h-cultured LC are remarkably different and are the most active accessory cells yet to be described for primary T-dependent responses (Figs. 2 and 3). Given these findings, we propose that the critical sensitization phase of the immune response is not the obligatory result of antigen presentation for which 12-h LC seem competent. As a corollary, if one uses T cells that have been primed *in situ*, or T cell clones and hybrids that have been chronically stimulated *in vitro*, then one complicates one’s capacity to separately analyze the sensitization and presentation aspects of immune stimulation.

We wondered what, in addition to antigen presentation, is required to initiate T cell function. Possibly the mature LC can form a lymphocyte-activating factor, such as IL-1. However, we have confirmed that fresh EC produce large amounts of IL-1 (14), so that a lack of IL-1 would not appear to explain the observed hypoactivity of fresh EC in T cell sensitization (4). Also, we have yet to detect IL-1 in spleen dendritic cells (our unpublished observations), which are immunologically as active as cultured LC. The capacity of mature LC to bind T cells in the absence of antigen (Fig. 5) could be the critical feature that is acquired during LC maturation in culture. Both lymphoid dendritic cells and cultured LC initiate the clustering of responding T cells during primary responses, while other Ia<sup>+</sup> cells do not (4, 6–9). Likewise, rat mesenteric lymph dendritic cells cluster T cells in the absence of antigen (15), suggesting that the gut as well as the skin can give rise to this type of accessory cell. With lymphoid dendritic cells,

antigen-independent clustering has been observed with unprimed and primed, Lyt-2<sup>-</sup> and Lyt-2<sup>+</sup> T cells, but primed lymphocytes cluster with 10 times fewer dendritic cells (7, 11). The consequences of this clustering remain to be pinpointed. The expression of lymphokine genes cannot be induced in purified T cells by lectins or mitogenic mAb alone (16). Additional inputs or so-called second signals are required, either from accessory cells or PMA. These two signals might be provided by the antigen-dependent and antigen-independent components of clustering. Clustering may also provide a stable microenvironment in which antigen and/or needed cytokines are applied for prolonged periods. For example, mitogenic lectins must be present for >12 h before T cells become committed to enter cell cycle (17).

If our *in vitro* findings are applicable *in situ*, LC would lack the capacity to induce primary responses until they matured in a fashion comparable to that observed during culture. Factors controlling this maturation are under study. They could entail products that are released locally during epidermal injury, inflammation, or deposition of contact allergens. Alternatively, LC may begin to mature whenever they exit from the epidermis and/or enter into afferent lymph. Such movements seem to be enhanced after the local application of antigens (18). Once sensitized lymphocytes have formed during an immune response, LC within the skin could serve as active APC without the need for further maturation (including the acquisition of antigen-independent clustering capacity). Given their accessibility *in situ*, the induction of LC stimulatory function could provide a novel adjunct to the design of immunization strategies.

### Summary

Langerhans cells (LC) are Ia<sup>+</sup> leukocytes that account for <2% of the cells in murine epidermal isolates. We purified LC by cell sorting to study their capacity to stimulate antigen-specific responses from unprimed and sensitized T cells. Sorting was performed after 12 or 72 h of epidermal culture, since our earlier work had indicated that LC became immunologically active during that time interval. At 12 and 72 h, the LC were uniformly and equally rich in the Ia glycoproteins that are recognized by helper T cells. At both time points, LC were comparable in their capacity to stimulate sensitized helper T lymphocytes, and would cluster the T cells in an antigen-dependent fashion at 4°C. However, 12-h LC did not sensitize T cells, as indicated by their inactivity in stimulating the primary MLR or antibody response, and they were unable to cluster T cells in an antigen-independent fashion at 37°C. The latter properties were acquired during 72 h of culture. As a result, the function of 72-h LC fully resembled that of lymphoid dendritic cells. We propose that the maturation of stimulatory function within the dendritic cell lineage represents an important control point in the induction phase of cell-mediated immunity.

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