Brief Definitive Report

Cytokine Gene Expression in Murine Epidermal Cell Suspensions: Interleukin 1β and Macrophage Inflammatory Protein 1α Are Selectively Expressed in Langerhans Cells but Are Differentially Regulated in Culture

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Summary

Epidermal Langerhans cells (LC) are considered direct yet immature precursors of dendritic cells (DC) in the draining lymph nodes. Although the development of LC into potent immunostimulatory DC occurs in vitro and has been studied in detail, little is known about their profile of cytokine gene expression. By using reverse transcriptase polymerase chain reaction analysis to screen 16 cytokines followed by Northern blotting for selected analysis, we determined the cytokine gene expression profile of murine LC at different time points in culture when T cell stimulatory activity is increasing profoundly. LC regularly expressed macrophage inflammatory proteins, MIP-1α and MIP-2, and interleukin 1β (IL-1β). Both MIPs were downregulated upon culture and maturation into DC, whereas IL-1β was strongly upregulated in culture. MIP-1α and IL-1β mRNA were found only in LC, but not in other epidermal cells. Apart from trace amounts of IL-6 in cultured LC, several macrophage and T cell products were not detected. The cytokine expression profile of LC thus appears distinct from typical macrophages. The exact role of the cytokine genes we found transcribed in LC remains to be determined.

Dendritic cells (DC) represent a distinct system of MHC class II positive leukocytes that function to initiate primary T cell responses (for review see reference 1). DC are found in nonlymphoid as well as in lymphoid tissues. Epidermal Langerhans cells (LC) are the most extensively characterized nonlymphoid DC (for reviews see references 2 and 3). Studies of LC suggest that DC in nonlymphoid organs are actually immature precursors of the DC in the lymphoid tissues. There is evidence that LC can leave the epidermis, move via the afferent lymph, and give rise to the DC found in the draining lymph nodes (for reviews see references 1 and 2). The maturation of LC into potent immunostimulatory DC can be studied in vitro, and has been shown to occur upon short-term culture in response to GM-CSF and IL-1 (for reviews see references 1 and 2). This in vitro model of LC maturation seems to closely mimic events in situ as changes that occur in vitro, notably the marked upregulation of expression of MHC class II products and the increase in stimulatory activity, also occur in vivo with transplantation (4). Although a good deal has been learned about the response of LC to exogenous cytokines (5), it has not been determined what range of cytokines LC themselves produce (for review see reference 6). It is only known that enriched fractions of human LC secrete IL-1 activity (7) and, after stimulation by LPS and PMA, TNF-α as well (8). In addition, it was demonstrated recently by depletion experiments that murine LC were the source of IL-1β mRNA in epidermal cell (EC) suspensions (9). Information on the cytokine pattern of LC is scarce because of the difficulties in purifying this trace (1–3% of all EC) cell population. Since this information is important to understand the function of LC, we have separately compared highly enriched LC with keratinocytes, using reverse transcriptase PCR for screening followed by Northern blotting for further analysis. This approach allowed us to study the cytokine gene expression profile of LC at various stages of their maturation into DC. Our major findings are that LC express a distinct profile of cytokine genes, and that these are regulated differentially in culture.

Materials and Methods

Mice. Specific pathogen-free BALB/c (H-2^d) mice (6–12-wk-old of both sexes) were obtained from Charles River Wiga GmbH (Sulzfeld, Germany).

Preparation of Purified LC and LC-depleted Fractions. EC suspensions (containing 1–3% LC) were prepared from ear skin and treated

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with anti-Thy1-mAb and rabbit C′ exactly as described (5). This treatment removes the dendritic TCR γ/δ bearing Thy-1$^+$ EC, as well as the majority of suprabasal keratinocytes that express low amounts of Thy-1 antigen, and results in an EC suspension containing about 15% LC (5). LC were further enriched by panning (see below) right away as well after 12 or 72 h of EC culture to obtain 0-, 12-, and 72-h LC, respectively. For culture, the anti-Thy1/C′-treated EC were plated at a density of 20×10^6 cells in 100-mm petri dishes (No. 3003; Falcon Labware Oxnard, CA) in 15-ml medium RPMI 1640 supplemented with 10% FCS ([56°C, 0.5 h; Seromed, Biochrom KG, Berlin, Germany]; 1 mM L-glutamine; 5×10^{-5} M 2-ME; and 50 μg/ml gentamicin sulfate). 12-h LC were obtained by panning directly from 12-h nonadherent fractions. To obtain 72-h LC, the nonadherent fractions, which contain virtually all the LC, were removed from the petri dishes after 72 h, and floated on dense albumin columns as described (5). LC were then panned from the floating fraction which contained all the LC at 50–80% purity.

To determine the cytokine gene expression of LC, we had to devise a strategy that would allow us to enrich LC to virtually 100% yet save the LC-depleted fractions for simultaneous analysis to control for inevitable minor crosscontamination. To this end we modified our panning technique (5) (yield: $8–10 \times 10^4$ fresh or cultured LC/2 mouse ears) taking into account that panned LC could be lysed for RNA preparation while still attached to the petri dish. EC fractions were, therefore, treated with two rather than just one mAb reactive with LC. We used anti-I-E^{k,d} (clone 14-4-4S, mouse IgG2a, HB32 from the American Type Culture Collection (ATCC, Rockville, MD) plus anti-I-A^{b,d} mAb (clone B 21-2, rat IgG2b, TIB 229 from the ATCC) or, alternatively, in some experiments, antileukocyte common antigen (clone M 1/9, rat Ig2a, TIB 122 from the ATCC). EC were then panned on petri dishes coated with nonspecies specific goat anti-mouse Ig for 30 min on ice and for 5 min at room temperature (without adding competing mouse or rat Ig which are needed to detach LC by washing [5]). Nonadherent cells were then removed by washing until careful monitoring under the inverted phase-contrast revealed that only LC were left behind. LC could be easily identified by their cell surface processes which appeared spread out on the petri dish. At most two non-LC among 100 cells were detectable. This degree of enrichment (>98% LC) was confirmed in pilot experiments where the attached cells were stained with FITC-anti-I-A^{b,d} to identify LC by immunofluorescence as well. The nonadherent fraction was transferred to another anti-mouse Ig-coated petri dish and panned for another 15 min at room temperature to remove any residual LC. Contaminating LC were not detectable by flow cytometry (staining with FITC-anti-I-A^{b,d} as described [5]).

Besides the non-LC fractions left after panning, we also used EC that were not Thy1-depleted for RNA isolation. Freshly prepared EC were either treated with isotype-matched control Ab and complement, or depleted of LC by anti-Ia/C′ treatment followed by panning, and studied right away or after 12–72 h of culture.

Preparation of RNA and cDNA. Total cellular RNA was isolated by the guanidinium isothiocyanate/cesium chloride method as described (5). LC were lysed while still attached to the petri dishes used for panning by adding lysis buffer (4 M guanidinium isothiocyanate, 5 mM sodium citrate, 0.5% *N*-lauroylsarcosine sodium salt, 100 mM 2-ME). Lysates were subjected to cesium chloride gradient centrifugation. After butanol-chloroform extraction and precipitation in ethanol, the concentration of RNA was measured by A_{260} absorption. 5 μg of total cellular RNA (with or without prior treatment with RNAse-free DNAse [Promega Corp., Madison, WI]) were reverse transcribed using a cDNA cycle kit (Invitrogen Co., San Diego, CA) in a final reaction volume of 50 μl. 2 μl of this cDNA reaction mixture (representing 200 ng transcribed RNA derived from 2×10^5 LC) were used for PCR amplification.

PCR Conditions. PCR primer pairs were purchased (Clontech, Palo Alto, CA) except for IFN-α (5′ primer: 5′-AATGACCTCC-ACCAGCAGCT-3′; 3-primer: 5′-TCTCAGGTACACAGTGATCC-3′; size of amplified fragment: 137 bp; kindly provided by Dr. R. Zawatzky, German Cancer Center, Heidelberg, Germany), IFN-β (5′-primer: 5′-GAAAAGCAAGAGGAAAGATT-3′; 3′-primer: 5′-AAGTCTTCGAATGATGAGAA-3′; amplified fragment: 165 bp; kindly provided by Dr. R. Zawatzky), and MIP-2 (5′-primer: 5′-TCTTCCTCGGGCACTCCAGA-3′; 3′-primer: 5′-GGACA-GCAGCCCAGGCTCCT-3′; amplified fragment: 379 bp; kindly provided by Dr. B. Sherry, The Rockefeller University, New York, NY). 2 μl of cDNA were amplified in a final volume of 50 μl in the presence of 1 μM final concentration of 5′ and 3′ primers, and PCR master mix (200 μM dNTPs, 1 U of Taq polymerase (Promega Corp.), 50 mM Tris-HCl (pH 8.5), 5 mM $MgCl_2$, 50 mM NaCl, and 2 mM DTT. The reaction mixture was overlaid with a drop of sterile paraffin oil, and the PCR was performed in a DNA thermal cycler (Thermocycler 60/2; Bio-Med, Theres, Germany) for 30 cycles (1 min denaturation at 94°C; 2 min annealing at 60°C; 3 min extension time at 72°C, and a delay time of 7 min for the last step). 16 μl of the reaction mix were removed and separated at 60 V for 2 h in 1% agarose in 1× TBE buffer containing 0.5 μg/ml ethidium bromide. 1 μg of a 1-kb and a 123-bp DNA ladder (BRL Gibco, Gaithersburg, MD) were run in parallel as molecular weight markers. An UV transilluminator was used to view and photograph the gels. Ten independent experiments were performed with comparable results. Before each PCR amplification of LC-derived cDNA, we performed positive controls using templates (Clontech) as well as a constant batch of RNA we had prepared from restimulated T cells and LPS-stimulated peritoneal macrophages. Negative controls were included with each experiment (no cDNA = no cDNA, but actin primers added; no RT = RNA "reverse transcribed" in the absence of reverse transcriptase plus IFN-α or actin primers added). Specificity of the amplified bands was validated by their predicted size. For those cytokine genes that were found expressed in LC we also performed the PCR procedure described by Dallman et al. (10) to demonstrate specificity and to ensure that at the 30 cycles we used we had not passed the plateau of amplification. To this end, 10 μl of cDNA were used in the PCR and 15 μl of the amplification mix were removed every five cycles from 15 or 20 up to 50 cycles. PCR products were analyzed by gel electrophoresis or dot-blotted to Hybond-N$^+$ membrane (Amersham Corp., Arlington Heights, IL) and probed using ^{32}P-end-labeled internal oligos [IL-1β: 5′-GAGCCTGTAGTGCAGCTGTCTAATGGGAAC-3′; IL-6: 5′-CTCCAGGTAGCTATGGTACTCCAGAAGACC-3′; MIP-1α: 5′-GACACCTGGCTGGGAGCAAAGGCTGCTGGT-3′; MIP-2: 5′-CAGGGTCTTCAGGCATTGACAGCGCAGTTC-3′].

Northern Blotting. To confirm the results obtained by PCR screening we performed Northern blotting (10–15 μg total cytoplasmic RNA per lane) exactly as described (5) using cDNA probes for murine IL-1β (provided by Dr. P. W. Gray, Genentech Inc., South San Francisco, CA [11]), IL-6 (provided by Dr. F. Lee, DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA [12]), MIP-1α (provided by Dr. G. J. Graham, The Beatson Institute for Cancer Research, Glasgow, UK) (13), and MIP-2 (provided by Dr. P. Tekamp-Olson, Chiron Co., Emeryville, CA [14]). For MIP-1α we also used ^{32}P-labeled RNA probes prepared from coding sequences of SCI/MIP-1α (13) using standard techniques. Northern blotting experiments require the use of 200 mouse

ears to obtain 10 μg of total cytoplasmic RNA from highly purified LC.

Results

PCR Analysis of Cytokine mRNA Expression in Langerhans Cells. By using the reverse transcriptase PCR technique we found that 4 out of 16 tested cytokines were detectable in LC-enriched (>98%) fractions. We reproducibly obtained strong IL-1β–specific bands with 72-h LC-fractions, whereas bands with 12- and 0-h LC were usually weaker and in some experiments were even absent in 0-h LC (compare Figs. 1 and 2). It is interesting that IL-1α was not detectable in LC (Figs. 1–3). IL-6 mRNA was not found in 0- or 12-h LC, whereas 72-h LC yielded IL-6–specific bands, which were, however, faint (Fig. 2) to barely detectable (Fig. 1). MIP-1α was regularly and clearly expressed only by fresh LC (Fig. 1). MIP-2–specific bands of variable intensity were detectable in 0- and 12-h as well as 72-h LC. Simultaneous analysis of LC-depleted fractions showed that IL-1β and MIP-1α mRNAs were usually absent, indicating that keratinocytes do not transcribe these cytokine genes. IL-6 was found in 0- and 12-h LC-depleted fractions but was not detectable in 72-h LC-depleted fractions. MIP-2 was variably expressed by non-LC fractions. As LC-depleted fractions obtained after panning represent only a subset of EC (see Materials and Methods), we also analyzed EC that had not been treated with anti-Thy1 and C′. Such EC (mainly keratinocytes) expressed the expected range of cytokines such as IL-1α, TNF-α, GM-CSF, etc. We found that any detectable IL-1β or MIP-1α–specific bands disappeared after removal of LC by anti-Ia/C′ treatment and panning, whereas MIP-2–specific bands were still present (data not shown). This demonstrated that LC even among unseparated EC are the only source of IL-1β and MIP-1α. MIP-2 mRNA was detectable by PCR in fresh as well as cultured EC even after depletion of LC. In contrast, IL-6 mRNA was found in 0- and 12-h EC even after LC depletion, but was not invariably detectable in cultured and LC-free EC (data not shown).

Northern Blot Analysis of Cytokine mRNA Expression in Langerhans Cells. As limiting amounts of RNA (10 μg LC-RNA/200 mouse ears) prohibited the use of RNase protection assays or poly(A$^+$) RNA, we used total cytoplasmic RNA and Northern blot analysis to extend our PCR data for selected cytokine mRNAs. Northern blotting demonstrated that IL-1β mRNA is massively upregulated in LC after culture (Fig. 4). We were unable to detect IL-6 mRNA expression in LC or EC by Northern blot analysis even after 6-d film exposure (Fig. 5 *A*). Northern blotting using a MIP-1α cDNA probe either yielded no signal or a very weak band with fresh LC (Fig. 5 *B*), yet MIP-1α mRNA was more clearly shown to be expressed by fresh LC when blots were probed with a ^{32}P-labeled RNA probe (Fig. 6). Freshly isolated LC contained much more MIP-2 mRNA than cultured ones (Fig. 5 *C*). LC-depleted EC expressed MIP-2, but less than either fresh or cultured LC (Fig. 5 *C*).

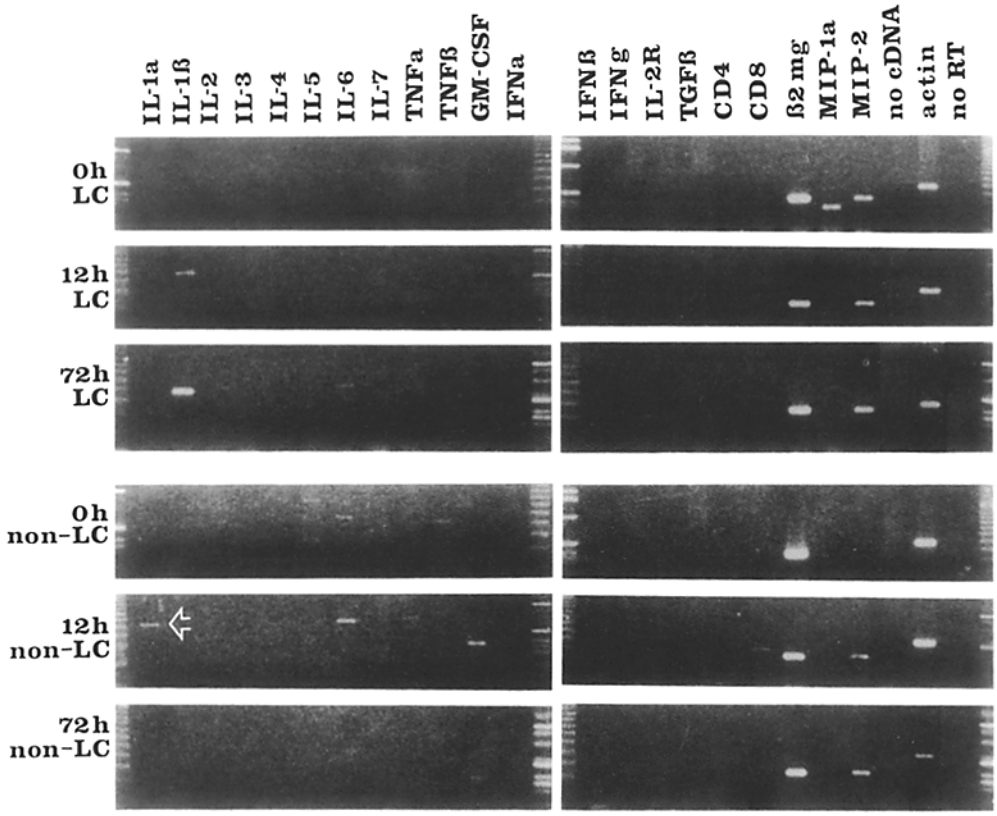

Figure 1. PCR analysis of cytokine gene expression in LC. LC were enriched (>98%) by panning from freshly prepared, 12- and 72-h cultured Thy-1–depleted EC (0-, 12-, and 72-h LC). RNA was prepared from LC-enriched and LC-depleted fractions (nonadherent fractions obtained after panning, non-LC), reverse transcribed and mRNA expression analyzed by PCR (IL-1α, IL-1β, IL-2–IL-7, TNF-α, TNF-β, GM-CSF, IFN-α, IFN-β, IFN-γ, IL-2 receptor, TGF-β, CD4, CD8, β2 microglobulin, MIP-1α, MIP-2, no cDNA control, actin, no RT control [see Materials and Methods]). Note that IL-1β mRNA signal appears in 12-h LC and is strong in 72-h LC, but is absent from non-LC. IL-1α is not expressed by LC fractions, but an IL-1α specific band (*open white arrow*) is expressed by 12-h non-LC. IL-6 is absent from LC except for a very faint band in 72-h LC. MIP-1α mRNA is clearly detectable in 0-h LC fractions, whereas 12-h LC display only a very faint band, and 72-h LC none at all. MIP-2 is expressed by all LC fractions. For further details see text.

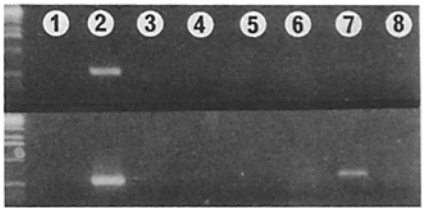

Figure 2. PCR analysis of cytokine gene expression in LC. These data from an experiment identical to the one outlined in Fig. 1 demonstrate the variability of IL-1β and IL-6 expression by LC. Note that in this experiment 0-h LC score clearly positive for IL-1β (lane *2*), and 72-h LC generate an unequivocal IL-6 signal (lane *7*) (compare with Fig. 1).

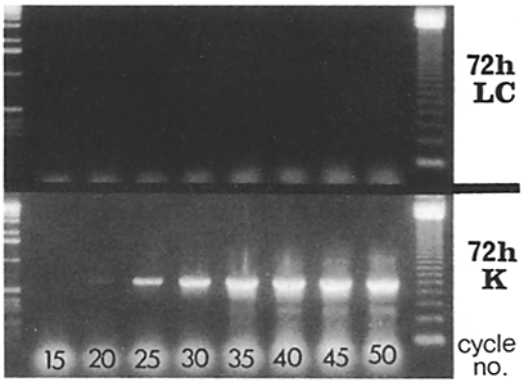

Figure 3. PCR-assisted mRNA amplification demonstrates absence of IL-1α in LC. cDNA samples were cycled through 50 cycles of PCR. 15 μl of the amplification mix were removed every five cycles and analyzed by gel electrophoresis. Note that 72-h LC-free EC (72-h keratinocytes) display an IL-1α–specific amplification fragment that is, however, absent from 72-h LC even at 50 cycles. Identical results were obtained with 0-h LC (data not shown).

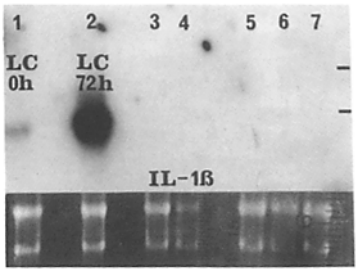

Figure 4. Northern blot analysis of IL-1β mRNA expression in LC and EC. Hybridization with a ^{32}P-labeled IL-1β cDNA probe (2-d film exposure) reveals some IL-1β mRNA in 0-h LC (lane *1*), much more in 72-h LC (lane *2*), but none in lanes *3* (0-h non-LC; see Fig. 1), *4* (72-h non-LC; see Fig. 1), *5* (fresh EC), *6* (fresh, LC-depleted EC), or 7 (LC-depleted EC cultured for 72 h). Ethidiumbromide-stained gels show integrity and loading (10 μg/lane) of RNA. (▬) 18 and 28S rRNA.

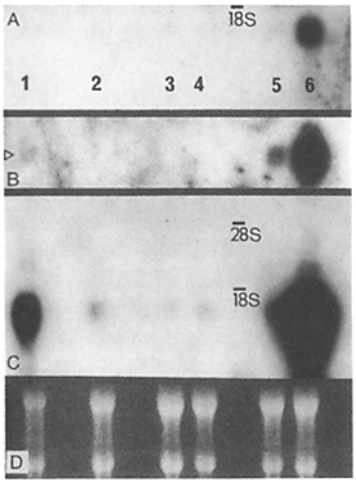

Figure 5. Northern blot analysis of IL-6, MIP-1α, and MIP-2 mRNA expression in LC and EC. The blot was probed with ^{32}P-labeled IL-6 cDNA (*A*), MIP-1α cDNA (*B*; *arrowhead* denotes barely visible MIP-1α transcript), and MIP-2 (*C*). Lanes *1*, 0-h LC; *2*, 72-h LC; *3*, 0-h non-LC (see Fig. 1); *4*, LC-depleted EC cultured for 72 h; *5*, peritoneal macrophages; and *6*, LPS-stimulated peritoneal macrophages (10 μg/ml, 8 h). (*D*) Ethidium bromide staining of gels (15 μg RNA/lane). Film exposure was for (*A*) 3 and 6 d (only 3 d is shown); (*B*) 3 and 6 d (only 6 d is shown); and (C) 3 d.

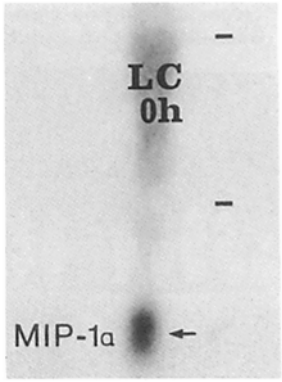

Figure 6. Northern blot analysis of MIP-1α mRNA expression in 0-h LC. 15 μg total cytoplasmic RNA derived from 0-h LC was hybridized with a ^{32}P-labeled MIP-1α–specific RNA probe. (←) marks the MIP-1α transcript. (▬) 18 and 28S rRNA.

Taken together, our data demonstrate that freshly isolated LC contain MIP-1α mRNA and substantial amounts of MIP-2 transcripts, whereas cultured LC express large amounts of IL-1β, yet little IL-6 mRNA. Only LC but not other EC (primarily keratinocytes) produce IL-1β and MIP-1α mRNA. LC undergo major changes in morphology, phenotype, and function when they develop into potent immunostimulatory DC during short-term culture (15). For example, antigen processing and T cell sensitizing functions are reciprocally expressed in fresh versus cultured LC (3, 16, 17). When we set out to study the cytokine gene expression of LC it was, therefore, crucial to examine LC at various stages of their maturation into DC. It also appeared essential to study highly enriched populations of LC, even though purification of this trace cell population is difficult, time-consuming and costly, rather than just simply to examine EC suspensions before and after depletion of LC. One reason was that only such an approach when combined with the highly sensitive PCR analysis would allow one to detect cytokine mRNAs present in low copy numbers and/or subsets of LC. Another reason

was that depletion studies would fail to identify LC as a source if the respective cytokine was expressed by LC as well as other EC. Indeed, in a recent study (9), only keratinocytes but not LC were thus identified as a source of MIP-2.

We found that four cytokine genes were expressed by LC and fell into two differentially regulated groups. Two of the cytokines, namely IL-1β and MIP-1α, were selectively expressed by LC but not by other EC. IL-1β appeared to be gradually but strongly upregulated during the 3-d culture period. IL-6 mRNA was detectable only in 72-h LC and only by PCR but not Northern blot analysis, which indicates that only few IL-6 transcripts were present. Recent in situ hybridization experiments using digoxigenin-labeled IL-6–specific antisense oligonucleotide probes indeed show that IL-6 is expressed only by a variable subset of 72-h LC (unpublished data). MIP-1α was weakly and MIP-2 quite strongly expressed by fresh LC, but both were downregulated upon culture. The kinetics of cytokine gene expression by LC is thus remarkably different from that of typical macrophages that one would not expect to differentially regulate the cytokines IL-1β, IL-6, MIP-1α and 2. Unlike macrophages, LC also did not express such genes as IL-1α, IFNα, and GM-CSF. We suspect that the LC cytokine gene expression profile that we observe during culture, particularly the gradual yet massive upregulation of IL-1β, is inherent to the maturation of LC into potent stimulatory DC, and not simply caused by exposure to trace amounts of LPS. In this context it is certainly notable that murine spleen DC, which like cultured LC are mature DC, lack IL-1α mRNA even after massive LPS stimulation (as shown by Northern blotting) (18), but express IL-1β (our own unpublished results) just like LC. Whether LC would express cytokine genes like IL-1α, IFNα, or GM-CSF after exogenous LPS stimulation has yet to be determined using approaches such as in situ hybridization. We tried to analyze the response of LC to LPS by PCR analysis. We were, however, unable to faithfully exclude that respective PCR signals in LC fractions were not due to small numbers of contaminating keratinocytes, as LPS caused a strong upregulation of cytokine gene expression in keratinocytes (unpublished data).

The translation of the four cytokines in LC, their expression in vivo, as well as their role, remain to be determined. The data obtained so far, however, already raise several interesting issues and provide a guideline for further studies. LC might participate in inflammatory reactions via secretion of IL-1β, IL-6, MIP-1α, and MIP-2, all of which represent proinflammatory cytokines. It will be particularly interesting to explore the potential roles of MIP-1α molecules. One intriguing possibility is that MIP-1α acts as a paracrine regulator of epidermal homeostasis since MIP-1α has recently been shown to act as an inhibitor of bone marrow (13) as well as keratinocyte stem cells (Parkinson, E. K., G. J. Graham, P. Daubersies, J. E. Burns, C. Heufler, M. Plumb, G. Schuler, and I. B. Pragnell, manuscript in preparation). It will also be interesting to assess whether MIP-1α modulates LC function inasmuch as MIP-1 peptides can act as autocrine modulators of their cell of origin (19).

The cytokine gene expression pattern which we find after preparation of EC and isolation of LC is likely induced relative to the epidermis in situ since we do not detect any cytokine mRNA by PCR analysis of heat-separated (55°C for 30 s), normal epidermis (unpublished data). As outlined in the introductory paragraph, the maturation of LC during culture of EC seems to closely mimic events in situ (4), and this seems to hold true for the cytokine gene expression profile of LC as well. In a recent study on the cytokine expression of EC in the induction phase of contact hypersensitivity (9), a massive upregulation of IL-1β was noted after epicutaneous application of contact allergens, but not after application of tolerogens, nonsensitizers, or irritants. LC were identified as the source of IL-1β mRNA amongst EC by depletion experiments. Our study now allows an interpretation of these interesting findings, since our data strongly suggest that the upregulation of IL-1β mRNA is actually due to the maturation of LC into DC during the induction phase of contact hypersensitivity. We are currently investigating how the maturation of LC into potent immunostimulatory DC is induced, and what role the massive expression of IL-1β might play for the function of LC.

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