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ORIGINAL ARTICLE

Control of the differentiation state and function of human epidermal Langerhans cells by cytokines *in vitro*

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ABSTRACT

Langerhans cells can originate *in vitro* from immature precursors stimulated with granulocyte macrophage-colony-stimulating factor (GM-CSF), tumour necrosis factor (TNF)-α and stem cell factor (SCF). We asked whether these cytokines also control the differentiation state of Langerhans cells within the epidermis and upon leaving this tissue. We harvested sheets of human epidermis by controlled dispase hydrolysis of keratomes, cultured them in RPMI and 10% fetal calf serum for 48 h and analysed the sheets and the cells migrated spontaneously into the medium, most of which were Langerhans cells containing Birbeck granules.[1] By flow cytometry, the intensity of CD1a expression was reduced quite evenly among Langerhans cells migrated from sheets within 48 h. The cells in the sheets underwent loss of dendrites, with a significant reduction in the cell perimeter that was prevented by GM-CSF and TNF-α together. Either of these cytokines induced expression of CD18 by cells in the sheets and those in the medium. Moreover, TNF-α induced expression of CD54 by cells in the medium, but not by those retained in the sheets, whereas human SCF induced, dose dependently, expression of CD54 by cells in the sheets, but not from those in the medium.[2] The proliferation of allogeneic lymphocytes was much higher when stimulating Langerhans cells were harvested from cultures with any cytokine, rather than from cultures without cytokines. We conclude the following: (i) GM-CSF and TNF-α help to maintain full differentiation of Langerhans cells within the epidermis; (ii) cytokine influence on Langerhans cells adhesiveness is in part context dependent; and (iii) pretreatment with cytokines influences positively the number or accessory activity of Langerhans cells on lymphocytes during subsequent mixed leucocyte reaction.

Key words: c-kit ligand, granulocyte-macrophage colony-stimulating factor, stem cell factor, tumour necrosis factor-α

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Introduction

Langerhans cells are part of a widely distributed system of dendritic cells specialized in antigen presentation. Resident epidermal Langerhans cells derive from CD34-positive myeloid precursors and their differentiation can be induced by many cytokines, including granulocyte-macrophage colony-stimulating factor (GM-CSF),[1,2] tumour necrosis factor (TNF)-α[2,3] and stem cell factor (SCF).[4,5] Langerhans cells are highly efficient in capturing and processing antigens; once loaded with antigens, they migrate out of the epidermis towards draining lymph nodes and in the meantime undergo a further differentiation step, by which they acquire a strong lymphocyte stimulating activity.[6,7] Although this step is often indicated as 'maturation', it is regulated by extrinsic clues and involves deep, irreversible changes in fine structure and antigen expression; therefore, it is in proper sense a process of differentiation.[8]

GM-CSF is a monomeric, globular glycoprotein;[9] it can be secreted by lymphocytes even in basal conditions and by many cell types upon stimulation; the latter include macrophages, endothelial cells, epithelial cells and fibroblasts.[10] This cytokine has been described to play a key part in the generation and

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viability of Langerhans cells *in vitro* and in the enhancement of their stimulatory capacity in a mixed leucocyte reaction.[2,11]

TNF-α is an acidic, unglycosylated protein; it is produced by monocytes/macrophages and other non-haematopoietic cell types, after appropriate stimulation.[12] This cytokine is also efficient in promoting the generation and maintaining the viability of Langerhans cells in culture.[2,3,13] The addition of GM-CSF and TNF-α to cultures of CD34-positive precursors, in the presence of serum, induces the differentiation of precursors into cells displaying the morphology and immunophenotype of Langerhans cells, including the presence of Birbeck granules and the expression of CD1a along with class II major histocompatibility complex molecules. The CD1a-positive cells harvested from cultures treated with GM-CSF and TNF-α induce a strong proliferation of allogeneic CD4-positive T cells in mixed leucocyte reaction,[2,3] although an inhibitory effect of this cytokine on tumour antigen presentation by Langerhans cells has been reported.[14]

Stem cell factor is a glycoprotein, synthesized as a transmembrane protein and produced by bone marrow stromal cells, T cells, keratinocytes, dermal cells and other cell types as well. The effects of this cytokine are mediated by a specific receptor, named c-kit, that has been recognized as a proto-oncogene before the identification of its ligand, SCF.[15] SCF has many biological activities, among which the ability to stimulate the proliferation of early haematopoietic progenitors.[4] In association with GM-CSF and TNF-α, SCF is able to augment the number of Langerhans and other dendritic cells obtained *in vitro*, while not affecting the morphological and immunophenotypical characteristics of such cells.[4,5,16–18]

In view of the role of GM-CSF, TNF-α and SCF in stimulating the differentiation of dendritic cells, including Langerhans cells, from immature precursors, the questions arise whether these cytokines are also relevant to maintain the differentiated state of Langerhans cells within the epidermis and whether they play any part in the further differentiation step that these cells undergo upon leaving the epidermis. We have addressed these issues in cultures of human epidermal sheets. We have analysed by immunohistochemistry and morphometry both the cells remaining within the epidermis and those migrated into the culture medium, and have assessed the accessory activity of the latter cells *in vitro*.

Material and methods

Epidermal sheets and isolated cells

Human epidermal sheets were obtained at plastic surgery from keratomes of normal, sun-unexposed human skin, in accordance with Italian law and the ethical standards of the Helsinki declaration. The epidermis was separated from the dermis with dispase (grade II; Boehringer Mannheim, Mannheim, Germany). Fresh cell suspension was obtained incubating the epidermis with trypsin for 1 h and was analysed by flow cytometry. Langerhans cells were obtained as described,[19] briefly: the epidermal sheets were further cut into pieces of about 15 mm^2 and placed into Petri dishes, floating on RPMI 1640 with the addition of 10% fetal calf serum, 10 U/mL penicillin, 10 mg/mL streptomycin and 50 mg/mL amphotericin B (all reagents for the culture medium were from Seromed, Milan, Italy); the cultures were incubated for 48 h at 37 °C in an atmosphere of air and 5% CO_2, in the following conditions: (i) with the addition of TNF-α (Pepro Tech, London, UK), 20 ng/mL; (ii) with the addition of GM-CSF (Pepro Tech), 0.1 ng/mL; (iii) with both TNF-α and GM-CSF, each at the same concentration as indicated above; (iv) with the addition of human SCF (Pepro Tech) at concentrations of 1, 5 and 10 ng/mL; (v) without any addition.

In preliminary experiments, doses of 40 and 80 ng/mL TNF-α and of 0.2 and 0.4 ng/mL GM-CSF were tested. As the results were always the same, the lowest doses, as indicated above, were used for the final experiments.

After 48 h the sheets were fixed in cold acetone for 10 min and kept in 0.1 mol/L phosphate-buffered saline (PBS), pH 7.4, calcium and magnesium free PBS, at 4 °C, until staining within 1 week.

The cells that migrated out of the sheets were collected from the medium and analysed by flow cytometry or cytospinned with the addition of 20% fetal calf serum to the culture medium; the cytospins were fixed for 3 min in cold acetone and stored at –20 °C until labelled.

Immunofluorescence

Spread epidermal sheets and cytospins were rehydrated, blocked with 5% normal calf serum and incubated with primary monoclonal antibodies (all from Immunotech, Marseilles, France) against CD1a, CD 18 (common β-chain of the adhesion molecules: lymphocyte function-associated antigen-1, Mac-1 and gp150,95), CD 54 (intercellular adhesion molecule-1) and CD 80 (adhesion molecule B7-1). This incubation was protracted for 90 min at 37 °C, and was followed by a fluorescein-isothiocyanate-conjugated goat antimouse antibody (Sigma, Milan, Italy) for 1 h at 37 °C. The slides were mounted with Gel/Mount (Biomeda, Foster City, California) and observed in a Zeiss (Oberkochen, Germany) Axioskop microscope equipped for epifluorescence.

To control for non-specific staining the appropriate isotype controls were used. To identify Langerhans cells, cells were double stained with HLA-DR and CD1a. All the incubations were done at 4 °C for 30 min.

Morphometry

The number of CD1a-labelled cells/mm^2 epidermis was evaluated directly at the microscope, on at least 3 mm^2 of epidermis. To evaluate the effects of treatments on the size and

shape of Langerhans cells, photomicrographs were taken of sheets labelled with CD1a and printed at final magnification $\times 400$. The profiles of immunostained cells were traced on a transparency that was overlaid on photomicrographs, and captured into a Macintosh Performa 6300 by a Color OneScanner 600/27 (Apple Computer, Cupertino, CA, USA); the area and perimeter of each cell profile were measured with Image 1.4 software (National Institute of Health, Bethesda, MD, USA). These parameters of cell profiles are directly correlated, respectively, to the volume and surface area of cells themselves.[20]

Electron microscopy

Electron microscopy was used to check that the enzymatic action of dispase did not alter basal keratinocytes and that cells migrated out of the sheets had the ultrastructural features of Langerhans cells. Therefore, in some experiments the sheets and migrated cells were fixed with a mixture of 2% formaldehyde and 2.5% glutaraldehyde in 0.1% mol/L cacodylate buffer, pH 7.4, at room temperature, osmicated and embedded in Epon 812. One micron thick sections were stained with toluidine blue and observed by light microscopy; 70 nm thick sections were stained with uranyl acetate, followed by bismuth tartrate or lead citrate and observed in a Siemens (Munich, Germany) 102 electron microscope, at 80 kV.

Flow cytometry analysis

Cells suspended from freshly prepared epidermis and cells collected from the medium after 48 h culture were stained and analysed with a FACS Calibur (Becton Dickinson Mountain view, CA). Antibody dilution and washing steps were done in PBS containing 2% fetal calf serum then 1×10^5 cells per reaction were stained with the following monoclonal antibodies at 10 μg/mL: CD1a phycoetythrin from Dako (Glostrup, Denmark) and HLA-DR fluorescein isothiocyanate from Becton Dickinson (Mountain View, CA, USA). In order to block Fc-mediated unspecific binding, cells were incubated with fetal calf serum and PBS (1 : 1) for 30 min at 4 °C. To control for non-specific staining the appropriate isotype controls were used. Langerhans cells were identified by double staining with HLA-Dr and CD1a. All incubations were done at 4 °C for 30 min.

Allogeneic mixed leucocyte reaction

Allogeneic, responder T lymphocytes were isolated from human peripheral blood of healthy donors, on a Lymphoprep gradient. The cells at the interface were collected and washed in PBS; three consecutive cycles of separation, collection and washing were performed. The last wash was in RPMI medium. The cells were counted, adjusted to 5×10^6 per mL and cultured for 90 min in Petri dishes at 37 °C with 5% CO_2. The non-adherent cells were harvested and more than 95% were pure T cells; they were washed for three consecutive passages in warm RPMI and counted again. They were cocultured with Langerhans cells, migrated out of the sheets within 48 h with or without cytokines, after a purification of these latter cells on Lymphoprep gradient (ICN Biomedicals, Aurora, OH, USA). For these experiments, GM-CSF and TNF-α were used at the concentrations indicated above and SCF at concentration 5 ng/mL. Langerhans cells were incubated with cytokines only during migration from the epidermis, i.e. before admixing with lymphocytes; no cytokine was added during mixed leucocyte reaction. The responder T cells and the stimulator Langerhans cells, in number of 9000 and 900, respectively, per well, were cocultured for 6 days in round-bottomed 96 wells microtitre plates (Nunc, Roskilde, Denmark). They were incubated in a total volume of 200 mL RPMI with 10% fetal calf serum. After 6 days coculture, the cells were pulsed with [^{3}H]thymidine (25 Ci/mmol, 1 mCi per well; Amersham International, Amersham, U.K.). The cells were then collected in a cell harvester and the incorporation of the isotope was measured in a liquid scintillation counter and expressed as the average count per minute of a triplicate.

Statistics

Data were subjected to analysis of variance. When this gave significant results, couples of values were further analysed with Student's *t*-test for paired values. $P < 0.05$ was assumed as significant in all cases. Results are given as mean ± SD.

Results

Epidermal sheets

Epidermal sheets were well preserved upon separation from the dermis and after 48 h culture as well. The number of CD1a-positive dendritic cells, i.e. Langerhans cells within the sheets was 392 ± 62 per mm^2 (mean ± SD of three experiments) at the onset of culture and decreased insignificantly with time (290 ± 127 per mm^2 upon 48 h without cytokines; results of three experiments). No treatment with cytokines affected significantly the number of Langerhans cells (data not shown). The number and length of dendrites also decreased with time in untreated sheets and this led to a decrease in the surface area and perimeter of Langerhans cells that was prevented at various extents by GM-CSF and TNF-α, even more with the cytokines together (fig. 1). However, the differences in cell area among experimental conditions were not shown to be significant by analysis of variance (fig. 2), whereas the differences in cell perimeter were shown to be significant ($P < 0.05$) and hence were further analysed in pairs (one-tailed test). This identified that the decrease during culture without cytokines was significant ($P < 0.05$) and that only a combined treatment with GM-CSF and TNF-α gave significantly better results ($P < 0.05$) than no treatment at all (fig. 3). CD1a antigen was expressed

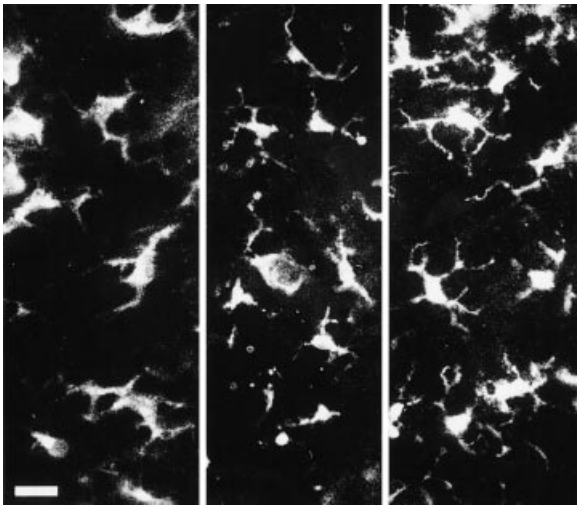

fig. 1 Langerhans cells were well preserved in freshly prepared epidermal sheets (left panel). The dendrites became shorter and less branched upon 48 h culture without added cytokines (central panel); these alterations were prevented by adding granulocyte-macrophage colony-stimulating factor (0.1 ng/mL) and tumour necrosis factor-α (20 ng/mL) to the culture medium (right panel). Indirect immunofluorescence for CD1a; scale bar, 15 μm.

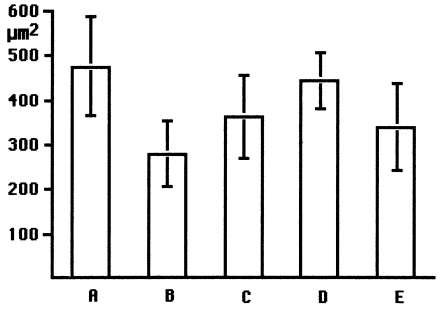

fig. 2 The surface area of CD1a cells in spread epidermal sheets is an estimate of the cell volume. It was reduced upon culture and this reduction was prevented by the addition of cytokines to the culture medium. However, the differences among experimental conditions were not significant. Experimental conditions were as follows: (A) start of culture; (B) 48 h culture without cytokines; (C) 48 h culture with granulocyte-macrophage colony-stimulating factor (GM-CSF) (0.1 ng/mL); (D) 48 h culture with GM-CSF (0.1 ng/mL) and tumour necrosis factor (TNF)-α (20 ng/mL); (E) 48 h culture with TNF-α (20 ng/mL).

with similar intensity, at all times and in all experimental conditions. The adhesion molecules CD18, CD54 and CD80 were not expressed in unstimulated sheets, either at the onset of culture or 48 h later. The expression of CD18 by Langerhans cells in the epidermis was induced by GM-CSF and TNF-α, either alone or associated with each other, whereas the expression of CD54 was stimulated by human SCF at higher doses, more precisely at 5 ng/mL in some cells and at 10 ng/mL in virtually all cells (Table 1).

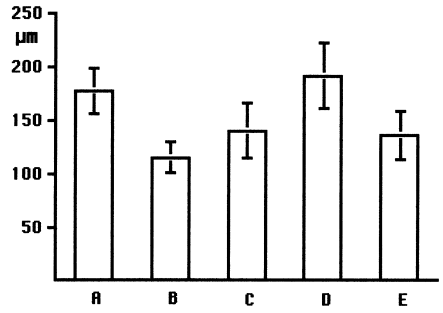

fig. 3 The perimeter of CD1a cells in spread epidermal sheets is an estimate of the cell surface area and hence of dendriticity. It was reduced significantly upon 48 h culture without cytokines ($P < 0.05$) and upon culture with granulocyte-macrophage colony-stimulating factor (GM-CSF) (0.1 ng/mL) or tumour necrosis factor (TNF)-α (20 ng/mL). This reduction was prevented by the addition of both GM-CSF and TNF-α to cultures, so that the cell perimeter in these conditions was significantly different from that upon culture without cytokines or with either cytokine alone ($P < 0.05$), but not significantly different from that at the start of culture. Experimental conditions are labelled as in fig. 2.

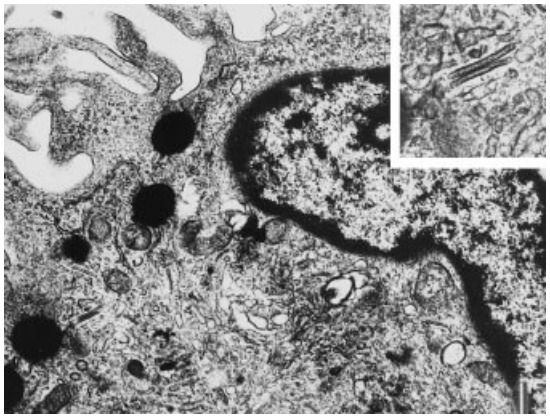

fig. 4 Cells migrated out of epidermal sheets during culture had a dendritic shape, were rich in organelles and contained Birbeck granules (inset), i.e. the marker of Langerhans cells. Electron microscopy; scale bar, 330 nm (130 nm for the inset).

Cells migrated into the culture medium

The vast majority of cells migrated out of the sheets and collected from the culture medium were Langerhans cells, as shown by CD1a staining analysed by fluorescence microscopy, electron microscopy and flow cytometry (figs 4, 5, 6b). Interestingly Langerhans cells migrated into the medium, although still CD1a positive, downregulated the intensity of CD1a expression if compared with the fresh cells (fig. 6a). The shape, adhesive properties and antigen expression of these cells were influenced by GM-CSF and TNF-α, but not by human SCF at any dosage (Table 1). In particular, the cells in the medium expressed CD18 in the presence of GM-CSF or

Table 1 Expression of membrane antigens by Langerhans cells in cultured epidermal sheets and by those migrated into the culture medium

			48 h culture						
							hSCF		
		Onset of culture	no cytokines	GM-CSF	TNF-α	GM-CSF + TNF-α	1 ng/mL	5 ng/mL	10 ng/mL
Epidermal sheets	CD1a	+	+	+	+	+	+	+	+
	CD18	−	−	+	+	+	−	−	−
	CD54	−	−	−	−	−	−	±	+
	CD80	−	−	−	−	−	−	−	−
Migrated cells	CD1a	+	+	+	+	+	+	+	+
	CD18	−	−	+	+	+	−	−	−
	CD54	−	−	−	+	+	−	−	−
	CD80	−	−	−	−	−	−	−	−

Human epidermal sheets were cultured in RPMI with 10% fetal calf serum, with or without cytokines as indicated. The concentration of GM-CSF was 0.1 ng/mL and that of TNF-α 20 ng/mL. After 48 h culture, the sheets and the cytospins of the cells migrated into the medium were analyzed by immunofluorescence. Positivity by all or by the vast majority of cells is indicated as +, positivity by a minority of cells is indicated as ±. The total amount of cells that could be expected to be labeled with each antibody was estimated in sheets by the cells labeled for CD1a, and in cytospins by the total cells in the slide.

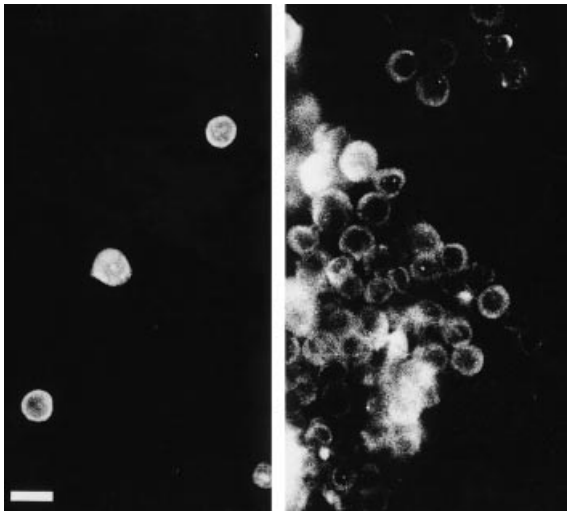

fig. 5 Cells migrated out of epidermal sheets during culture and analysed in cytospins expressed CD1a and were isolated from each other upon granulocyte-macrophage colony-stimulating factor (left panel) as well as in unstimulated conditions. Upon tumour necrosis factor-α (20 ng/mL) they aggregated into clusters and expressed CD54, i.e. intercellular adhesion molecule-1 (right panel). Indirect immunofluorescence for CD1a (left) and CD54 (right); scale bar, 15 μm.

TNF-α; furthermore, they aggregated into large clusters and expressed CD54 in the presence of TNF-α (fig. 5).

Allogeneic mixed leucocyte reaction

The Langerhans cells migrated out of the sheets within 48 h culture maintained their functional activity of stimulating the proliferation of T lymphocytes in a mixed leucocyte reaction. The cells isolated from cultures treated with cytokines were all significantly stronger stimulators of lymphocyte proliferation than those isolated from control

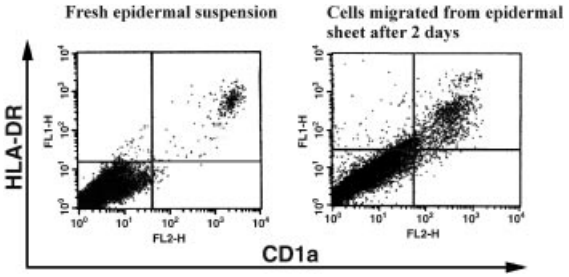

fig. 6 Flow cytometry analysis for HLA-DR and CD1a double-stained fresh (a) and migrated Langerhans cells (b); in panel b the cells show down-regulation of CD1a antigen, if compared with fresh ones (panel a), but this antigen is still expressed by most cells.

cultures not treated with cytokines ($P < 0.05$; two-tailed test). Among cytokines, GM-CSF provided the greatest enhancement of the accessory activity of Langerhans cells; however, the differences among cytokines in this respect were not significant (fig. 7).

Discussion

In this paper we have shown the following:

1 A combination of GM-CSF and TNF-α prevented the shrinkage and loss of dendrites of human Langerhans cells in cultured epidermal sheets.
2 GM-CSF and TNF-α, but not human SCF, stimulated the expression of CD18 containing adhesion receptors by Langerhans cells in the sheets and by those migrated into the culture medium.
3 The expression of intercellular adhesion molecule-1 by Langerhans cells in the sheets was stimulated by human SCF in a dose-dependent way and the expression of this adhesion molecule by cells migrated into the culture medium was stimulated only by TNF-α.

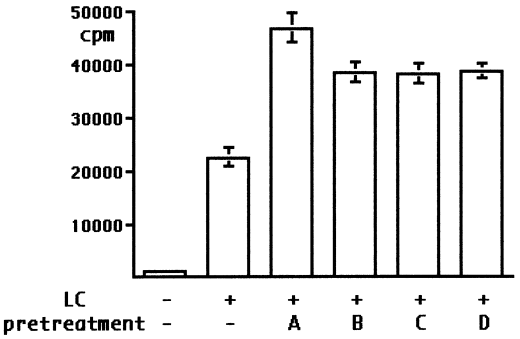

fig. 7 The cells harvested from the culture medium were cocultured with allogeneic lymphocytes and the proliferation of the latter cells was estimated by the incorporation of [3H]thymidine as measured in a liquid scintillation counter; the results are expressed as counts per minute (cpm). Lymphocytes with no added cells were used as negative controls. The cells harvested from untreated cultures stimulated lymphocyte proliferation significantly ($P < 0.05$). Treatment with cytokines further increased significantly the stimulatory capacity of harvested cells ($P < 0.05$). LC: Langerhans cells. Pretreatments of Langerhans cells before coculture with lymphocytes, i.e. during migration from epidermal sheets for 48 h, were as follows: (A) granulocyte-macrophage colony-stimulating factor (GM-CSF) (0.1 ng/mL); (B) GM-CSF (0.1 ng/mL) and tumour necrosis factor (TNF)-α (20 ng/mL); (C) TNF-α (20 ng/mL); (D) stem cell factor (5 ng/mL).

4 The Langerhans cells migrated into the culture medium were potent accessory cells for allogeneic lymphocytes and this activity was increased by GM-CSF, TNF-α and human SCF.
5 The downregulation of CD1a by CD1a migrated out of epidermal sheets within 48 h culture depends prevalently on reduced expression of antigen by single cells, rather than on a decrease in number of positive cells.

The association of GM-CSF and TNF-α proved to be necessary and effective to maintain fully developed dendrites to the cells retained in the sheets. This indicates that apart from their direct action in inducing the differentiation of immature precursors into Langerhans cells,[2] these cytokines are effective in maintaining a full-blown differentiated state of Langerhans cells in the epidermis.

With the methods used in this study, CD18 appeared to be not expressed by unstimulated Langerhans cells, either in the epidermis or upon migration into the culture medium. On the contrary, the expression of CD18 in both conditions was markedly stimulated by GM-CSF and TNF-α at the doses used in these experiments. This molecule is part of adhesion receptors, including the counter receptor for CD54, i.e. lymphocyte function-associated antigen-1. The stimulation of CD18 expression was specific, as another cytokine, namely human SCF was ineffective in this respect, whereas the latter cytokine influenced the expression of another adhesion molecule, CD54. Previous reports on the role of SCF on Langerhans cells differentiation have stressed the influence of this cytokine on immature precursors, rather than on already differentiated cells, and all have included preceding or contemporary treatment with other cytokines besides SCF.[4,5,17] Therefore, our finding that medium to high doses of human SCF alone stimulate CD54 expression by Langerhans cells in the epidermis cannot be compared with previous reports and indicate that this cytokine can influence fully differentiated Langerhans cells in the epidermis, although only in a limited respect (i.e. CD54 expression). This finding can represent the consequence of a direct influence of human SCF on epidermal Langerhans cells. However, as only high doses were effective and as Langerhans cells migrated into the culture medium did not respond to SCF with expression of CD54, an indirect effect mediated through keratinocytes can be considered as an alternative explanation for our findings, as keratinocytes express the receptor for SCF[21] and can in turn secrete cytokines stimulating Langerhans cells.[22]

The expression of CD54 by the cells migrated out of the sheets only in the presence of TNF-α suggests that this cytokine stimulates a progression towards a further differentiation step of Langerhans cells. This interpretation is based on the facts that CD54 is expressed by Langerhans cells upon activation and is necessary for stimulatory interactions with lymphocytes.[7,23–25] A major finding of this study was that the stimulation of Langerhans cells with TNF-α is context sensitive. On one side, cells free in the culture medium responded to TNF-α, either alone or in association with GM-CSF, by increasing reciprocal stickiness and expressing the adhesion molecule CD54, as already described by others.[26] On the other side, cells in the epidermis responded to TNF-α when it was associated with GM-CSF, by maintaining well developed dendrites during culture. Keratinocytes could provide an inhibitory signal for the expression of adhesion molecules in response to TNF-α by secretion of soluble molecules or by direct contact with Langerhans cells. Keratinocytes can secrete several cytokines potentially affecting Langerhans cells and interfering with TNF-α.[22] Also, keratinocytes can establish contacts with Langerhans cells mediated by E-cadherin[27,28] and intercellular contacts can themselves influence the cell behaviour under several respects.[29,30]

The negativity for CD80 antigen of cells in the sheets and of those in the medium contrasts with other data from the literature;[31] however, in those experiments dendritic cells had been obtained from immature precursors and had been treated with TNF-α for 24 h after having been stimulated with GM-CSF and interleukin-4. Therefore, those data cannot be compared strictly with ours, that indicate that 48 h stimulation with TNF-α are not enough to induce Langerhans cells from the human epidermis to express CD80 upon leaving the epidermis itself and migrating into a culture medium.

The results of mixed leucocyte reaction showed that the cells harvested from the culture medium had accessory activity towards T lymphocytes and this activity was stimulated by exposure to cytokines before coculturing with lymphocytes. During coculture, both Langerhans cells and lymphocytes deliver each other signals through soluble and membrane bound molecules, that stimulate both types of cells.[24,32–34] This might explain why Langerhans cells become potent accessory

cells even in the absence of any previous treatment with cytokines, as is well known from the literature.[6,7,35] The responses of the cells harvested from GM-CSF and TNF-α treated media in this study are in line with what is known from the literature for Langerhans cells.[2,3] In addition, our results indicate that SCF affects human Langerhans cells in their ability to stimulate T lymphocytes, similar to TNF-α and insignificantly less than GM-CSF; however, it did not influence the morphology and immunophenotype of these cells as long as they were in the culture medium without lymphocytes (at variance with GM-CSF and TNF-α, which both induced the expression of CD18 and, TNF-α, also induced the expression of CD54). The effects of cytokine preincubation of Langerhans cells on lymphocyte proliferation might depend on cytokines enhancing the viability of Langerhans cells and preventing their apoptosis,[36,37] thus allowing for a higher number of stimulating cells in mixed cultures.

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