

TUMOR NECROSIS FACTOR  $\alpha$  MAINTAINS THE VIABILITY  
OF MURINE EPIDERMAL LANGERHANS CELLS IN  
CULTURE, BUT IN CONTRAST TO  
GRANULOCYTE/MACROPHAGE COLONY-STIMULATING  
FACTOR, WITHOUT INDUCING THEIR  
FUNCTIONAL MATURATION

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Dendritic cells (DC)<sup>1</sup> are class II-positive leukocytes specialized to initiate primary T cell-dependent immune responses (1-3). DC were first identified in lymphoid tissues, and most of the studies that have unraveled their unique stimulatory capacity for resting T cells used DC isolated from lymphoid organs. New insight into the biology of DC came from studies of murine epidermal Langerhans cells (LC) in vitro (4-14). These studies suggest that LC in the skin and possibly DC in other nonlymphoid tissues as well represent precursors or immature elements of the DC system (15, 16). Resident LC constitutively express class II MHC antigens, but are only weak stimulators of resting T cells when isolated from the skin. During 2-3 d of bulk epidermal cell (EC) culture, however, LC increase their sensitizing activity for resting T cells 10-30-fold, and come to resemble lymphoid DC in morphology and surface markers as well (4). Granulocyte/macrophage colony-stimulating factor (GM-CSF) was identified as the principal mediator of this LC maturation in vitro (6, 7). GM-CSF maintains LC viability *and* increases function, whereas IL-1 enhances LC function twofold when combined with GM-CSF but does not support viability by itself (7).

As LC in situ are immature but presumably long-lived (17-19), we have now searched for a cytokine that would keep LC alive *without* inducing their functional maturation. Among a panel of purified cytokines tested, only TNF- $\alpha$  exhibited this activity. This finding reveals yet another facet of this pleotropic mediator (20, 21). Whether TNF- $\alpha$  plays any physiological role in LC homeostasis is unknown at present, but appears possible as we found TNF- $\alpha$  mRNA in freshly prepared EC.

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<sup>1</sup> *Abbreviations used in this paper:* DC, dendritic cells; EC, epidermal cells; GM-CSF, granulocyte/macrophage CSF; hu, human; mu, murine.

### Materials and Methods

*Mice.* Specific pathogen-free BALB/c (H-2<sup>d</sup>) and C3H/He (H-2<sup>k</sup>) mice (6–12 wk old of both sexes) were obtained from Charles River Wiga GmbH, Sulzfeld, FRG, and C3H/HeJ mice were from Bolmholtgard Breeding and Research Centre Ltd., Ry, Denmark.

*Culture Medium.* The medium was RPMI 1640 supplemented with 10% FCS (56°C, 0.5 h; Seromed, Biochrom KG, Berlin, FRG), 1 mM L-glutamine,  $5 \times 10^{-5}$  M 2-ME, and 50  $\mu$ g/ml gentamicin sulfate.

*Preparation of EC Suspensions.* EC suspensions were prepared from ear epidermis as described (4), except that 1% trypsin (40 min) was used for processing the ventral, thick ear halves, and 0.6% (20 min.) for the dorsal, thin ear halves.

*Identification of LC.* LC were identified by staining with FITC-conjugated anti-I-A<sup>b,d</sup> mAb (clone B21-2, rat IgG2b, TIB 229, from the American Type Culture Collection [ATCC], Rockville, MD; the mAb was purified from culture supernatants and conjugated to FITC by standard methods; as an isotype-matched control mAb we used FITC-anti-Thy-1, Becton Dickinson & Co., Mountain View, CA) and/or by phase-contrast microscopy (LC in contrast to contaminating keratinocytes exhibit cell surface processes, which are short and fine on fresh LC, and large and long on cultured LC) (4, 5). For evaluation by fluorescence microscopy staining was performed on cells that were cytopun (Shandon Cytospin 2; Shandon Labor-technik GmbH, Frankfurt, FRG) onto glass slides or attached to poly-L-lysine-coated multiwell slides. For evaluation by flow cytometry (using a FACStar instrument; Becton Dickinson Immunocytometry Systems, Mountain View, CA), cells were stained in suspension. Propidium iodide was added at a final concentration of 0.5  $\mu$ g/ml to stain dead cells and exclude them from analysis by software gating.

*LC Enrichment from Freshly Prepared EC Suspensions.* Freshly prepared BALB/c EC suspensions (viability >85%; containing 1–3% LC) were treated with anti-Thy-1 mAb (culture supernatant from clone 13.4, mouse IgM, TIB99 from ATCC) and low-tox-M rabbit complement (Cedarlane Laboratories, Hornby, Ontario, Canada) at a final concentration of  $3 \times 10^6$  cells/ml. This treatment removed dendritic Thy-1<sup>+</sup> EC as well as the majority of keratinocytes that express low amounts of Thy-1 (22). The cell suspension (viability 10–20%) was washed twice with cold PBS without calcium and magnesium (Seromed), treated for 10 min at 37°C with 0.125% trypsin (No. 16-893-49; Flow Laboratories) at  $10^6$  cells/ml in the presence of 80  $\mu$ g/ml DNase I (Sigma Chemical Co., St. Louis, MO), and finally washed again. This procedure removed most dead cells and resulted in a viable (>90%) EC suspension containing ~15% (range 10–28%) LC. LC were then further enriched by either panning, or, in some experiments by FACS.

*LC Enrichment by Panning.* This was performed as described (6). Briefly, after treatment with anti I-E<sup>k,d</sup> (clone 14-4-4S, mouse IgG2a, HB32 from ATCC), the cells were panned on petri dishes coated with goat anti-mouse Ig. The nonadherent cells were washed off, and the attached, LC-enriched cells were eluted by pipetting in the presence of mouse Ig. The populations thus obtained consisted of 76–92% LC. The yield was  $1-2 \times 10^4$  LC/mouse. For sorting the cell suspensions were stained with FITC-anti-I-A<sup>b,d</sup> (clone B21-2, see above). Cell sorting was performed on a FACS III instrument (Becton Dickinson & Co., Sunnyvale, CA) at 3,000 cells/s. The fluorescent fraction was >90% pure.

*Culture of Enriched LC.*  $10^5$  fresh LC enriched by either panning or sorting were plated in 16-mm wells (No. 3424; Costar Europe, Badhoevedrop, The Netherlands) in 1 ml medium with or without purified cytokines. After 72 h, and in a few experiments also after 24 or 36 h, the nonadherent cells were removed, the wells were rinsed twice with 1 ml medium, and the pooled cells were washed twice. Then the percentage and viable yields of LC were determined and their accessory function was tested.

*Purified Cytokines, Anti-GM-CSF Antibody.* For information on the panel of cytokines we used, see Table I. Rabbit anti-murine GM-CSF serum (23) was kindly provided by Dr. S. Gillis (Immunex Corp., Seattle, WA). A 1% vol/vol dose neutralized 20 ng/ml of recombinant murine GM-CSF (Table II).

*LC Enrichment from 72-h bulk EC Cultures.*  $20 \times 10^6$  freshly prepared BALB/c EC were plated in 100-mm petri dishes (No. 3003; Falcon Labware, Oxnard, CA) in 15 ml medium.

TABLE I  
Cytokines Tested on Epidermal Langerhans Cells

Cytokine	Expressed in:	Activity*	Dose	Source
mu rIL-1	<i>E. coli</i>	$6 \times 10^6$	10 ng/ml	P. LoMedico, Hoffman-La-Roche, Nutley, NJ
hu rIL-2	<i>E. coli</i>	$3 \times 10^6$	100 U/ml	Amersham, Buckinghamshire, UK
mu IL-3	Natural	$10^7$	100-1,000 U/ml	Genzyme Co., Boston, MA
mu rIL-4	Yeast	$10^8$	100-1,000 U/ml	Genzyme
mu rIL-5	Baculovirus	$10^5$ U/ml	10-100 U/ml	W. Strober, NIAID
hu rIL-6	Yeast	$10^7$	10-1,000 U/ml	Genzyme
mu rIL-7	cos7sup	$1.5 \times 10^4$ U/ml	250 U/ml	S. Gillis, Immunex Co., Seattle, WA
hu rG-CSF	<i>E. coli</i>	$10^8$	100-1,000 U/ml	Amersham
hu rM-CSF	Yeast	$10^8$	100-1,000 U/ml	Genzyme
mu M-CSF	Natural	$8 \times 10^7$	100-1,000 U/ml	R. Stanley, Albert Einstein College of Medicine, Bronx, NY
mu rGM-CSF	Yeast	$4 \times 10^7$	0.1-2.0 ng/ml	S. Gillis
mu IFN- $\alpha$	Natural	$2 \times 10^6$	200 U/ml	Stratech Scientific Ltd., London, UK
mu IFN- $\beta$	Natural	$1.2 \times 10^8$	100 U/ml	Stratech Scientific Ltd.
mu rIFN- $\gamma$	CHO cells	$1 \times 10^8$	10-100 U/ml	Holland Biotechnology, Leiden, NL
mu rTNF- $\alpha$ †	<i>E. coli</i>	$2.6 \times 10^7$	31-500 U/ml	G. R. Adolf, Bender Co., Vienna, Austria
mu rTNF- $\alpha$ §	<i>E. coli</i>	$4 \times 10^7$	31-500 U/ml	Genzyme
hu rTNF- $\alpha$	<i>E. coli</i>	$6 \times 10^7$	100-5,000 U/ml	G. R. Adolf
hu rTNF- $\alpha$	<i>E. coli</i>	$2 \times 10^7$	100-5,000 U/ml	Genzyme
mu EGF	Natural	—	5-200 ng/ml	Flow Lab., Meckenheim, FRG
mu FGF	Natural	—	100-200 ng/ml	Flow Lab., Meckenheim, FRG
mu NGF- $\beta$	Natural	—	100 ng/ml	Boehringer Mannheim, Vienna, Austria
mu NGF 7S	Natural	—	100 ng/ml	Boehringer Mannheim
hu rPDGF	CHO cells	—	2-100 ng/ml	Amersham
TGF- $\beta$ (porcine)	Natural	—	0.1-10 ng/ml	R&D Systems, Inc., Minneapolis, MN

\* U/mg unless otherwise stated.

†§ Both rTNFs gave similar results, but † was used in most experiments.

After 72 h nonadherent cells were removed and floated on dense albumin columns as described (4). The floating fraction contained all the LC at 40-75% purity.

*T Cell Proliferative Assays.* To test the stimulatory capacity of LC for unprimed T cells, we used the polyclonal response of periodate-modified T cells ("oxidative mitogenesis") and the primary MLR as described (4). LC were irradiated (900 rad from a  $^{137}\text{Cs}$  source) before addition to the assays. T cells were nylon-wool nonadherent, anti-Ia/complement-treated spleen and mesenteric lymph node cells, which in part of the experiments were further purified by an additional step involving sedimentation in discontinuous Percoll gradients (45-54-63%) (8) in which dead cells and remaining accessory cells floated.

*Preparation of Total Cellular RNA from EC.* Total cellular RNA was isolated from freshly prepared as well as cultured (3 and 24 h) EC by the guanidinium isothiocyanate/cesium chloride method (24). Cells in suspension ( $\sim 30 \times 10^6$ ) were washed in PBS, centrifuged, and then resuspended in 5 ml of lysis buffer (4 M guanidinium isothiocyanate, 5 mM sodium citrate, 0.5% *N*-lauroylsarcosine sodium salt, 0.1 M 2-ME). Adherent cells were first rinsed, and then lysed by adding lysis buffer to the petri dishes. Lysed cells were loaded on a cesium chloride density gradient (1.5 ml of 5.7 M cesium chloride, 1 ml each of 40, 30, and 20% wt/vol cesium chloride), and centrifuged in a Sorvall TH 641 rotor at 36,000 rpm at 18°C

TABLE II  
GM-CSF As Well As Murine TNF- $\alpha$  Support the Survival of LC

Additives to culture medium		Recovery of viable LC after 72 h in culture
None		4.0 $\pm$ 2.6 (10)
mu rGM-CSF	0.1 ng/ml	31.6 $\pm$ 4.7 (3)
	0.5 ng/ml	48.4 $\pm$ 3.8 (3)
	1.0 ng/ml	48.2 $\pm$ 4.3 (3)
	2.0 ng/ml	48.1 $\pm$ 5.8 (30)
mu rGM-CSF	0.5 ng/ml	
+ anti-GM-CSF	0.0250% vol/vol	3.5 $\pm$ 2.3 (4)
+ anti-GM-CSF	0.0125% vol/vol	26.5 $\pm$ 4.9 (2)
mu rTNF- $\alpha$	31 U/ml	21.8 $\pm$ 9.9 (5)
	62 U/ml	45.4 $\pm$ 6.5 (15)
	125 U/ml	46.8 $\pm$ 4.8 (15)
	250 U/ml	42.4 $\pm$ 7.1 (7)
	500 U/ml	45.7 $\pm$ 7.8 (7)
Anti-GM-CSF (0.5% v/v)		
+ mu rTNF- $\alpha$	62 U/ml	48.7 $\pm$ 4.5 (10)
+ mu rTNF- $\alpha$	125 U/ml	49.0 $\pm$ 4.6 (10)
mu rGM-CSF (2 ng/ml)		
+ m rTNF- $\alpha$	62 U/ml	53.3 $\pm$ 9.7 (10)
+ m rTNF- $\alpha$	125 U/ml	50.3 $\pm$ 5.1 (5)
hu rTNF- $\alpha$	100 U/ml	4.5 $\pm$ 2.1 (3)
	500 U/ml	5.7 $\pm$ 3.8 (3)
	1,000 U/ml	19.0 $\pm$ 4.5 (3)
	5,000 U/ml	16.7 $\pm$ 3.8 (3)
All other cytokines in Table I		<10% (3)

$10^5$  LC enriched by either panning (>75%) or sorting (>90%) were cultured in 1 ml medium  $\pm$  additives. After 72 h, viable LC yields were determined and expressed relative to the number of LC at the start (100%). Data are means  $\pm$  SD with the number of experiments in parentheses.

for 18 h. The pellet containing total cellular RNA was resuspended in 0.1% SDS in diethylpyrocarbonate-treated water and extracted three times with an equal volume of 1-butanol/chloroform (1:1) and once with chloroform. RNA was precipitated with 0.1 vol of 3 M sodium acetate pH 5 and 2.2 vol of 100% ethanol overnight at  $-20^\circ\text{C}$ , pelleted by centrifuging at 12,000  $g$  for 10 min, resuspended in water, and then stored at  $-80^\circ\text{C}$ .

**cDNA Probes.** Murine GM-CSF cDNA probe (cloned into pgem3; 0.88 kb; Bam HI/Eco RI insert; provided by Dr. N. M. Gough [25]), murine TNF- $\alpha$  cDNA probe (in pUC9; 1.3 kb; Pst I/Bam HI insert; provided by Dr. A. Cerami, The Rockefeller University, New York, NY), murine IL-1 $\alpha$  clone pIL1 1301 (1.7 kb; Bam HI/Bam HI insert; provided by Dr. P. LoMedico [26]), mouse m $\beta$ 5 tubulin cDNA (in pUC9; 1.6 kb; Eco RI/Eco RI insert; provided by Dr. Don W. Cleveland [27]), and chicken  $\beta$ -actin cDNA (in pBR322; 1.9 kb; Hind III/Hind III insert; provided by Dr. D. W. Cleveland [28]) were labeled with  $\alpha$ - $^{32}\text{P}$ dATP (Amersham International, Amersham, UK) using the oligoprimmer procedure (29) to a specific activity of  $1-5 \times 10^9$  cpm/ $\mu\text{g}$  DNA.

**RNA Blots.** The purified RNA was quantitated by  $A_{260}$  and analyzed by electrophoresis in 1% agarose/6% formaldehyde gels followed by blot transfer to Hybond N nylon membranes (Amersham). Blots were hybridized overnight with  $10^6$  cpm/ml at  $65^\circ\text{C}$  and washed for 30 min each in  $3 \times \text{SSC}/1\% \text{SDS}$ ,  $1 \times \text{SSC}/1\% \text{SDS}$ ,  $0.3 \times \text{SSC}/0.1\% \text{SDS}$ , and  $0.1 \times \text{SSC}/0.1\% \text{SDS}$  at  $65^\circ\text{C}$ . Filters were exposed 1-4 d to Cronex film with intensifying screens.

## Results

*Murine TNF- $\alpha$  Supports the Survival of Enriched LC in Culture.* In accordance with previous results we found that enriched LC (>75% by panning or sorting; results were comparable with LC enriched by either method) die when cultured in medium alone, but survive when GM-CSF is included (GM-CSF reached plateau activity at 0.5 ng/ml, and was routinely used at 2.0 ng/ml) (Table II). When we tested a panel of additional cytokines we found that one other cytokine, namely murine TNF- $\alpha$ , supported LC viability as well. Maximal activity of murine rTNF- $\alpha$  was reached at 62 U/ml (Table II). It was, therefore, used at 62 or 125 U/ml in all further experiments. The observed TNF effect on LC viability did not appear to be due to the induction of GM-CSF production during the assay as it was not abolished by the addition of rabbit anti-mouse GM-CSF serum in doses up to 0.5% vol/vol, which blocked 10 ng GM-CSF/ml (Table II). Surprisingly, human TNF- $\alpha$  was not active at comparable doses, and therefore, this particular TNF activity exhibited a species preference. At higher doses (see Table II) human TNF partially supported LC viability, but we have not studied whether the induction of GM-CSF was responsible for this effect. LC cultured in GM-CSF, when viewed under an inverted phase-contrast microscope, exhibited many large dendritic and veiled processes as described previously (4). The cell surface processes of LC cultured in murine TNF- $\alpha$  were less prominent (not shown) and similar to the ones found on LC isolated from bulk EC cultured overnight (5). LC did not proliferate in culture as demonstrated by [<sup>3</sup>H]thymidine autoradiography (data not shown).

*Enriched LC When Cultured in TNF- $\alpha$  Are Weak Stimulators of Resting T Cells in Contrast to LC Cultured in GM-CSF.* We have described previously that freshly isolated LC are weak stimulator cells for resting T cells (4-6). In accordance with preceding studies (6, 7), we found that enriched LC when cultured in GM-CSF (plateau effect at 0.5 ng/ml) for 72 h matured into potent stimulator cells (see Table II) and exhibited a similar activity as LC that had matured in companion bulk EC cultures. LC cultured for 72 h in 62 or 125 U/ml murine TNF- $\alpha$ , however, were 10-30-fold less active at limiting doses (Tables III-VI). The low stimulatory capacity was reflected by the formation of only few accessory cell/T cell clusters during the assay (Fig. 1). LC cultured in TNF alpha *plus* GM-CSF developed stimulatory activity, which indicated that the poor activity of LC cultured in TNF- $\alpha$  alone was not due to a toxic effect of TNF alpha (Table IV). The low stimulatory activity was also not due to induction and carryover of a suppressive factor, as the addition of conditioned media did not alter the potent stimulatory activity of LC enriched from bulk EC cultures (Table IV) or of LC cultured in GM-CSF (not shown). LC cultured in TNF- $\alpha$  when added to the assays also did not suppress T cell proliferation induced by LC enriched from bulk EC cultures (not shown). We next tested the activity of LC cultured in either GM-CSF or TNF- $\alpha$  at 24 or 36 h to exclude that LC when cultured in TNF- $\alpha$  acquired potent but transient stimulatory activity early on. The respective experiments (Table V and VI) demonstrated that LC cultured in GM-CSF progressively increase their stimulatory activity, whereas those cultured in TNF- $\alpha$  remain weak from the beginning. When LC were cultured for 36 h in TNF- $\alpha$ , and then GM-CSF was added to the wells, stimulatory activity developed (Table VI). This demonstrated that TNF- $\alpha$  did not irreversibly block LC maturation.

As LC cultured in TNF- $\alpha$  or GM-CSF differed in stimulatory capacity we were

TABLE III  
*LC Cultured for 72 h in TNF- $\alpha$  Are Weak Stimulators (Oxidative Mitogenesis Assay) in Contrast to LC Cultured in GM-CSF*

Stimulator cells	[ <sup>3</sup> H]TdR uptake with doses of			
	$3 \times 10^3$	$1 \times 10^3$	$3 \times 10^2$	$1 \times 10^2$
	<i>cpm</i> ( $\times 10^{-3}$ )			
LC, enriched from 72-h bulk EC cultures	131.0	81.0	27.0	6.0
Panned LC, cultured 72h:				
in GM-CSF	108.0	59.0	18.0	5.0
TNF- $\alpha$	17.0	4.0	1.4	0.6

LC were enriched to 82% by panning, cultured for 72 h in culture medium supplemented with 2 ng/ml mu rGM-CSF or 125 U/ml mu rTNF- $\alpha$ . Then their stimulatory function for  $3 \times 10^5$  periodate-modified (syngeneic BALB/c) T cells was tested (oxidative mitogenesis assay) and compared with LC isolated from companion 72-h bulk EC cultures (enrichment to 60% by flotation on dense albumin columns). T cells alone took up <1,800 cpm.

interested to see whether there was a difference in expression of class II MHC antigens as well. From previous work we know that during LC maturation in bulk EC culture there is about a fivefold increase in surface class II molecules that reaches plateau levels more quickly than the stimulating activity of LC (12-18 versus 60-72 h) (5, 11). Enriched LC cultured in TNF- $\alpha$  or GM-CSF for 72 h exhibited comparable

TABLE IV  
*LC Cultured for 72 h in TNF- $\alpha$  Are Weak Stimulators (Primary Allogeneic MLR) in Contrast to LC Cultured in GM-CSF. The Low Stimulatory Activity Is Not Due to a Toxic Effect or Induction of a Suppressive Factor.*

Stimulator cells	[ <sup>3</sup> H]TdR uptake with doses of			
	$1 \times 10^4$	$3 \times 10^3$	$1 \times 10^3$	$3 \times 10^2$
	<i>cpm</i> ( $\times 10^{-3}$ )			
Sorted LC, cultured 72h in:				
(a) TNF- $\alpha$	7.0	5.0	0.5	0.4
(b) TNF- $\alpha$ + GM-CSF	70.0	55.0	35.0	18.0
(b) GM-CSF	71.0	54.8	37.0	19.0
LC, enriched from 72h bulk EC cultures	ND	55.0	30.0	25.00
+ 12.5% conditioned medium a	ND	80.0	35.0	20.0
+ 12.5% conditioned medium b	ND	75.0	35.0	21.0

LC were enriched to >90% by FACS, cultured for 72 h in culture medium supplemented with 2 ng/ml mu rGM-CSF, 62 U/ml mu rTNF- $\alpha$ , as well as GM-CSF + TNF- $\alpha$ . Then their stimulatory function for  $3 \times 10^5$  C3H T cells was tested (primary allogeneic MLR). Stimulatory activity was compared with LC enriched (60%) from companion 72-h bulk EC cultures by flotation on dense albumin columns. T cells alone took up <400 cpm. Note that LC cultured in TNF, but not LC cultured in GM-CSF with or without TNF are weak stimulators. Note also that the addition of conditioned medium taken from LC cultured for 72 h in TNF (a) is not suppressive.

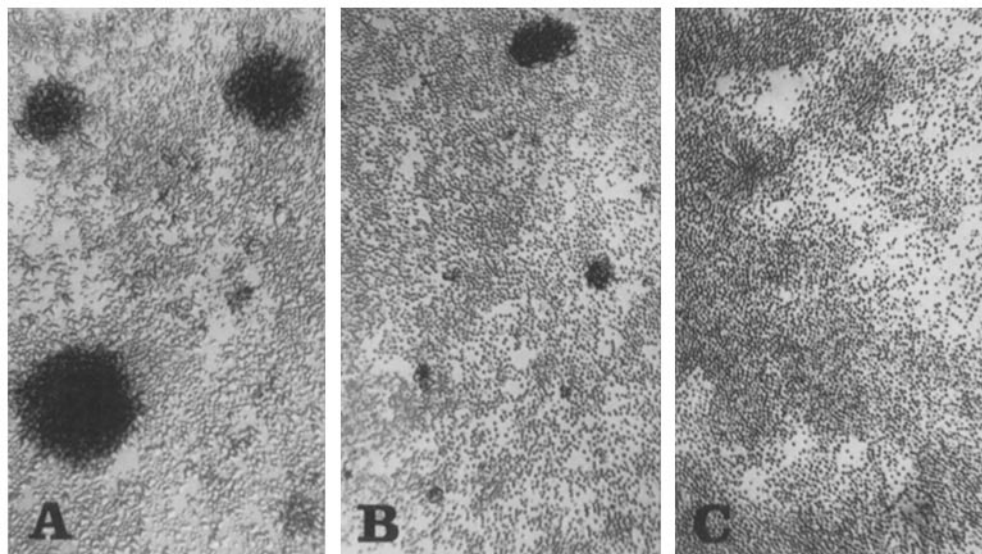


FIGURE 1. Cluster formation during T cell proliferative responses as observed under the inverted phase-contrast microscope. The pictures were taken from an experiment as outlined in Table III. The wells containing top doses of stimulators were photographed at the time of harvesting (i.e., 40 h after onset). Many large clusters and T blasts are seen with LC cultured for 72 h in GM-CSF as stimulators (A), but not with LC cultured in TNF- $\alpha$  (B). T cells without LC added are small and round, and neither clusters nor blasts are present (C).

TABLE V  
LC Cultured in TNF- $\alpha$  Are Weak Stimulators  
Throughout the Culture Period of 72 h

Stimulator cells	[ <sup>3</sup> H]TdR uptake with doses of			
	$3 \times 10^3$	$1 \times 10^3$	$3 \times 10^2$	$1 \times 10^2$
	<i>cpm</i> ( $\times 10^{-3}$ )			
Sorted LC no. 1, cultured 24h in				
(a) GM-CSF	83.3	43.8	23.1	8.6
(b) TNF- $\alpha$	21.8	12.3	3.2	1.5
(c) TNF- $\alpha$ + anti-GM-CSF	17.8	9.3	3.5	1.1
Sorted LC no. 2, cultured 72h in				
(a) GM-CSF	180.7	126.4	72.1	33.1
(b) TNF- $\alpha$	37.6	19.8	5.1	2.7
(c) TNF- $\alpha$ + anti-GM-CSF	38.0	10.9	4.0	1.7

Two LC populations (both enriched to >90% by FACS) were cultured for 24 (LC no. 1) and 72 h (LC no. 2), respectively, in culture medium supplemented with 2 ng/ml mu rGM-CSF, 62 U/ml mu rTNF- $\alpha$ , as well as mu rTNF- $\alpha$  + anti-GM-CSF (0.5%). LC nos. 1 and 2 were harvested at the same time and their stimulatory function for  $3 \times 10^5$  periodate-modified (syngeneic BALB/c) T cells was tested (oxidative mitogenesis assay). Stimulatory capacity was compared with LC enriched to 60% from companion 72-h bulk EC cultures. T cells alone took up <800 cpm.

TABLE VI  
*The Low Stimulatory Activity of LC Cultured in TNF- $\alpha$*   
*Is Quickly Reversed by GM-CSF*

Stimulator cells	[ <sup>3</sup> H]TdR uptake with doses of			
	3 × 10 <sup>3</sup>	1 × 10 <sup>3</sup>	3 × 10 <sup>2</sup>	1 × 10 <sup>2</sup>
	<i>cpm (× 10<sup>-3</sup>)</i>			
Panned LC, cultured in				
GM-CSF (36 h)	99.2	34.7	14.0	4.0
TNF alpha (36 h)	20.2	6.7	1.6	0.8
GM-CSF (72 h)	147.0	105.6	41.5	15.2
TNF alpha (72 h)	34.3	12.3	3.2	1.3
(36 h), then + GM-CSF	156.1	69.7	34.4	9.0

LC were enriched to 84% by panning, and cultured for 36 as well as 72 h in 2 ng/ml mu rGM-CSF or 125 U/ml rTNF- $\alpha$ . In addition, part of the LC was first cultured in TNF alone for 36 h, and then GM-CSF was added to the culture wells. The stimulatory activity for 3 × 10<sup>5</sup> periodate modified (syngeneic BALB/c) T cells was tested (oxidative mitogenesis assay) after 36 as well as 72 h of culture. T cells alone took up <1,100 cpm.

levels of I-A antigens when studied by flow cytometry (Fig. 2). The fact that enriched LC survive for 24 h even in plain medium (6) enabled us to determine whether the upregulation of class II antigens would occur even if no exogenous cytokines were added. After 24 h of culture surface I-A antigens had reached the plateau levels found on LC cultured for 72 h under all the conditions tested, i.e., when fresh LC were cultured in medium alone, in medium supplemented with neutralizing rabbit

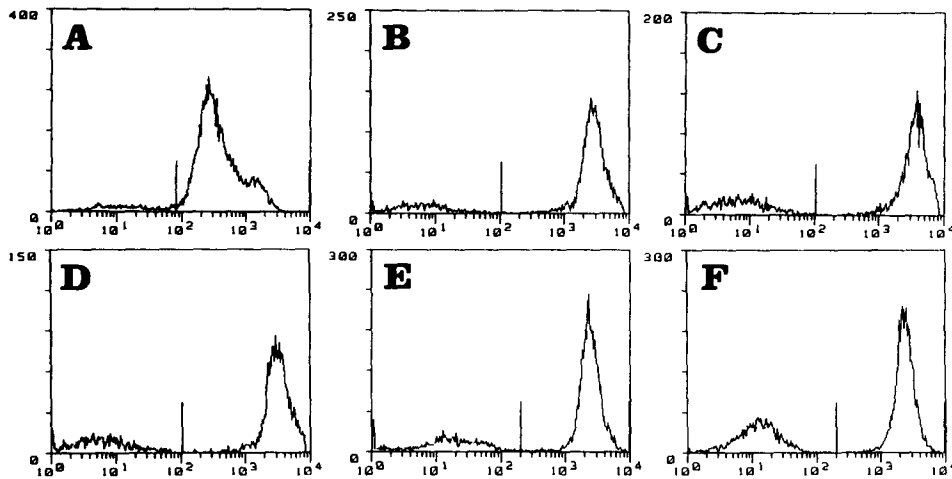


FIGURE 2. Cell surface levels of Ia antigens on fresh and cultured LC as assessed by flow cytometry. Cells were stained with a saturating dose of FITC-mAb to I-A<sup>b,d</sup> (clone B21-2) or with an isotype-matched FITC anti-mouse IgM control (not shown) to determine background staining (indicated by vertical bar). (A) LC freshly isolated by panning; (B) LC cultured for 24 h in medium + anti-GM-CSF (0.5%); Ia staining was identical with LC cultured 24 h in medium alone, or in medium supplemented with GM-CSF or TNF- $\alpha$  (data not shown). LC cultured for 72 h in GM-CSF (C), TNF- $\alpha$  (D), and in TNF- $\alpha$  + GM-CSF (E); LC enriched from companion 72-h bulk EC cultures (F). The enrichment of LC was 92% (A), 83% (B), and 75% (C-E).

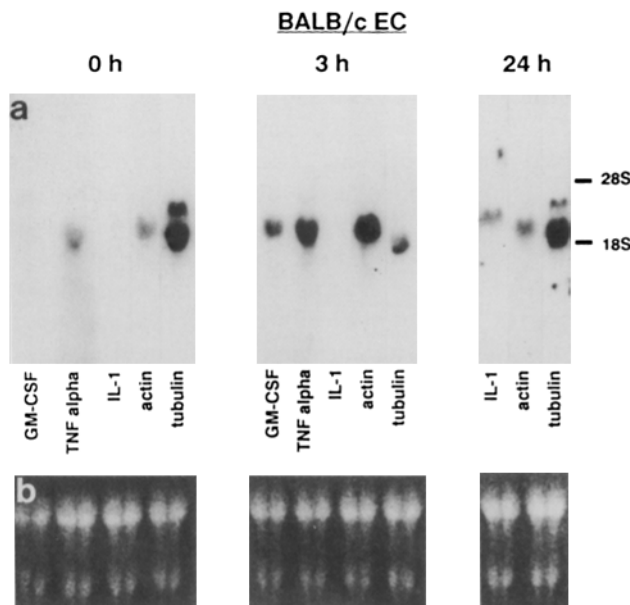


anti-mouse GM-CSF, GM-CSF, or TNF- $\alpha$ . The mechanism for this upregulation of class II antigens, therefore, has yet to be identified.

**TNF- $\alpha$  mRNA Is Expressed by Freshly Prepared EC.** To get a clue as to whether our in vitro findings could have any relevance for LC in the epidermal microenvironment we examined whether EC express mRNA specific for TNF- $\alpha$ , GM-CSF, and IL-1 $\alpha$ . Freshly prepared EC in several experiments expressed TNF- $\alpha$  mRNA, less or no GM-CSF mRNA, and no detectable IL-1 mRNA (Fig. 3). Interestingly, when tested after 3 h of culture, i.e., under conditions when LC maturation is known to occur, EC expressed significant levels of GM-CSF, and at 24 h IL-1 mRNA as well (Fig. 3A). Freshly prepared EC from LPS-responsive C3H/He and LPS-unresponsive C3H/HeJ mice showed comparable expression of TNF- $\alpha$  mRNA (data not shown).

### Discussion

When we searched for a cytokine that would keep highly enriched LC alive during 72 h of culture, but unlike GM-CSF, would not induce their potent stimulatory activity for resting T cells, we were surprised that among the panel of purified cytokines tested murine TNF- $\alpha$  exhibited such an effect (plateau at 62 U/ml; Tables II-VI). It was interesting, that *human* TNF- $\alpha$  was not active at comparable doses (Table II). This particular TNF- $\alpha$  activity, therefore, exhibited a species preference similar to that recently described for the growth factor effect of murine TNF- $\alpha$  on thymocytes (30) and a T cell line (31). After our initial observation, several concerns immediately arose. First, we had to exclude that the survival and low stimulatory activity of LC were simply due to the induction of insufficient amounts of GM-CSF by TNF- $\alpha$ . In agreement with previous studies (6, 7), we found that as little as 0.5 ng/ml of GM-CSF was sufficient for maximal recovery as well as functional maturation of highly enriched LC during 72 h of culture. The finding that culturing LC in TNF- $\alpha$



**FIGURE 3.** Northern blot analysis of cytokine (TNF- $\alpha$ , GM-CSF, IL-1- $\alpha$ ) mRNA expression in BALB/c EC. Total cellular RNA (20  $\mu$ g/lane) from BALB/c EC cultured for 0, 3, and 24 h was electrophoresed, transferred to Hybond N nylon membranes, and hybridized with the respective <sup>32</sup>P-labeled cytokine cDNA probes (2 d film exposure), and as a control, with actin and tubulin cDNA probes (6 h film exposure) (a). Note that even though the amounts of RNA per lane were constant (see b: ethidiumbromide stained 1% agarose/6% formaldehyde gels before transfer), the expression of actin and tubulin is not, and therefore, it appears to be regulated. One lane was used to probe actin and tubulin.

plus rabbit anti-GM-CSF in doses neutralizing as much as 10 ng/ml GM-CSF did not alter LC recovery or function (Tables II and V) as compared with culturing LC in TNF- $\alpha$  alone indicated that the effect of TNF- $\alpha$  was not due to the induction of GM-CSF. Second, we had to exclude that the low stimulatory capacity of LC cultured in TNF- $\alpha$  was caused by a toxic or a suppressive effect. When LC were cultured in TNF- $\alpha$  plus GM-CSF, or first in TNF- $\alpha$  and then for another 36 hours in GM-CSF, stimulatory function developed (Table VI). This indicated that the low stimulatory capacity of LC cultured in TNF- $\alpha$  alone was not simply due to a toxic effect of this cytokine, and that TNF- $\alpha$  did not block the maturation of LC mediated by GM-CSF. The addition of conditioned medium taken from LC cultured in TNF- $\alpha$  to the T cell proliferative assays did not alter the potent stimulatory capacity of purified LC cultured in GM-CSF or of LC enriched from 72 h bulk EC cultures (Table IV). This showed that the low stimulatory capacity of LC cultured in TNF- $\alpha$  was not due to the induction of a suppressive factor. The question whether other cytokines are intermediate to the effect of TNF- $\alpha$  on LC remains unsolved at present. The finding that none of the many cytokines tested except GM-CSF and TNF- $\alpha$  supported LC viability (Table II) indicates that TNF- $\alpha$  is indeed functioning directly. It does not exclude, however, that a combination of the cytokines we tested or some other mediator is induced and responsible for the observed effect.

We then had to ask whether TNF- $\alpha$  could at all play a role for LC homeostasis in vivo. The available data allow neither to firmly support nor to dismiss such a provocative hypothesis. A condition sine qua non would be that EC express TNF- $\alpha$  in situ. We found that freshly prepared EC contain TNF- $\alpha$  mRNA, but this does not necessarily mean that TNF- $\alpha$  mRNA is constitutively transcribed and translated in situ. Recent immunohistochemical studies of human epidermis suggest, however, that TNF- $\alpha$  protein might indeed be present in normal epidermis (32). Recently it was shown that cultured human keratinocytes can produce and release TNF- $\alpha$ , even though the constitutive production was found to be low (33). This is also true for murine keratinocytes, as in conditioned media of subconfluent keratinocyte monolayers cultured for 24 h TNF activity did not exceed 7 U/ml and often was not unequivocally detectable at all in the standard L929 assay (unpublished results). This does not a priori exclude that TNF- $\alpha$  might reach a concentration in situ at which the effect on LC that we observed in vitro could occur. In our in vitro culture experiments we added TNF- $\alpha$  at 62 U/ml just once at the beginning to LC that had undergone trypsinization and panning. If TNF- $\alpha$  had been added continuously and had acted upon unmodified LC, the concentration needed to keep LC viable might have been lower. One should also take into account that in situ even a low constitutive TNF- $\alpha$  release into the narrow, synapse-like intercellular space should result in substantial local concentrations. Recently, a transmembrane form of TNF- $\alpha$  (34) has been described that seems to be active through cell to cell contact and thus allows a localized action. Therefore, yet another possibility would be that a membrane-bound form of TNF- $\alpha$  is expressed by keratinocytes and mediates a paracrine effect on LC in the epidermal microenvironment. In future studies it will be necessary to monitor cytokines as well as cytokine receptors in situ under various conditions to substantiate if indeed a network of cytokines acts in concert to regulate the viability and function of LC in vivo as suggested by the in vitro studies outlined here and described previously (6, 7).

### Summary

Freshly isolated murine epidermal Langerhans cells (LC) are weak stimulators of resting T cells but increase their stimulatory capacity 10–30-fold upon 2–3 d of culture together with other epidermal cells. This maturation of LC is mediated by two keratinocyte products. Granulocyte/macrophage colony-stimulating factor (GM-CSF) maintains viability *and* increases function. IL-1 alone does not keep LC alive, but when combined with GM-CSF further enhances their stimulatory activity.

We have now searched for a cytokine that would keep LC in a viable, but functionally immature state. When LC (enriched to >75%) were cultured in the presence of GM-CSF (2 ng/ml) or murine (TNF- $\alpha$ ) (plateau effect at 62 U/ml), the recovery of viable LC after 72 h was identical. The LC cultured in murine TNF- $\alpha$ , however, were 10–30 times less active in stimulating resting T cells. A series of experiments demonstrated that this phenomenon was not due to the induction of insufficient amounts of GM-CSF, the induction of a suppressor factor, or a toxic effect of TNF- $\alpha$ . Interestingly, the observed TNF- $\alpha$  activity exhibited a species preference, as human TNF- $\alpha$  was not active at comparable doses.

We have observed an unexpected effect of TNF- $\alpha$  on LC *in vitro*. Though we found that freshly prepared epidermal cells express TNF- $\alpha$  mRNA, further studies are needed to establish whether TNF- $\alpha$  plays a role *in vivo* by keeping resident LC in a viable, but functionally immature state.

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