Phagocytosis of Antigens by Langerhans Cells In Vitro

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Summary

Dendritic cells (DC) isolated from lymphoid tissues are generally thought to be nonphagocytic in culture. It has therefore been unclear how these cells could acquire particulate antigens such as microorganisms for initiation of primary immune responses. Lymphoid DC derive in part from cells that have migrated from nonlymphoid tissues, such as Langerhans cells (LC) of skin. The ability of LC to internalize a variety of particles was studied by electron, ultraviolet, phase, and differential interference contrast microscopy, and by two-color flow cytometry. Freshly isolated LC in epidermal cell suspensions phagocytosed the yeast cell wall derivative zymosan, intact Saccharomyces cerevisiae, representatives of two genera of Gram-positive bacteria, Corynebacterium parvum and Staphylococcus aureus, as well as 0.5–3.5-μm latex microspheres. During maturation in culture, the phagocytic activity of these cells was markedly reduced. Likewise, freshly isolated splenic DC were more phagocytic than cultured DC for two types of particle examined, zymosan and latex beads. Unlike macrophages, LC did not bind or internalize sheep erythrocytes before or after opsonization with immunoglobulin G or complement, and did not internalize colloidal carbon. The receptors mediating zymosan uptake by LC were examined. For this particle, C57BL/6 LC were considerably more phagocytic than BALB/c LC and exhibited a reproducible increase in phagocytic activity after 6 h of culture followed by a decline, whereas this initial rise did not occur for BALB/c LC. These differential kinetics of uptake were reflected in the pattern of zymosan binding at 4°C, and endocytosis of the soluble tracer fluorescein isothiocyanate–mannose–bovine serum albumin at 37°C. Zymosan uptake by LC from both strains of mice was inhibited in the presence of mannan or β-glucan, although to different extents, but not by antibodies specific for CR3 (CD11b/CD18). These data indicate that zymosan uptake by LC can be mediated by a mannose/β-glucan receptor(s) that is differentially expressed in the two strains of mice and that is downregulated during maturation of LC in culture.

Members of the dendritic cell (DC)[1] family play a central role in the initiation of antigen-specific adaptive immune responses (1, 2). DC isolated from lymphoid organs (lymphoid DC) present foreign peptide–MHC complexes to resting T cells and deliver costimulatory signals for T cell activation that cannot be provided by most other cell types. There is accumulating evidence that DC in nonlymphoid tissues, such as Langerhans cells (LC) of skin epidermis, are functionally immature but that maturation commences as these cells migrate into secondary lymphoid tissues to become lymphoid DC (3). Freshly isolated (immature) LC have little

costimulatory activity (4) but they can endocytose and process native soluble antigens (5). LC develop into cells resembling mature lymphoid DC when they are cultured in the presence of cytokines, particularly GM-CSF (1, 2, 4). During maturation LC lose the capacity to process soluble antigens, acquire costimulatory activity, and undergo phenotypic changes (e.g., downregulation of CDw32 Fc receptors and upregulation of B7/BB1; type 3 complement receptors, CD11b/18, are retained). There is also a marked reduction in the biosynthetic rate of MHC class IIα, β, and invariant chains by LC, and a loss of acidic organelles, particularly early endosomes and Birbeck granules (1, 2, 4–9).

DC at some stage can acquire soluble antigens delivered in vivo, such as contact-sensitizing agents applied to the skin, proteins delivered in aerosolized form to the lung and airways, and antigens administered into the gut. These cells, and splenic

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[1] Abbreviations used in this paper: DC, dendritic cell; EC, epidermal cell; LC, Langerhans cell; Mph, macrophage; MR, mannose receptor; Strp, streptavidin.

DC pulsed with antigens in vitro, can induce immunity after adoptive transfer to naive recipients (1, 9–14). Soluble tracers have also been visualized within LC in situ after intradermal or intravaginal administration (15–18), and after incubation of LC in vitro (7). The phagocytic capacity of DC has been considerably less clear. The general consensus is that isolated lymphoid DC and veiled cells (VC) of afferent lymph are non-phagocytic in vitro, but there are contradictory reports that particles can be acquired by DC in vivo and occasionally in vitro (19–25). For adaptive immune responses to be mounted against pathogenic organisms, such as bacteria and yeasts, it would seem necessary that DC should be phagocytic at some stage in their life cycle. We have therefore studied the phago-cytic capacity of freshly isolated compared to cultured LC and splenic DC, as prototypes for immature and mature members of the DC lineage. Our observations indicate that phagocytosis is a specialized function of immature DC and that the phagocytic potential of these cells is markedly reduced during maturation.

Materials and Methods

Experimental Animals. Male C57BL/6J, BALB/c, and C3H/HeN mice were from Harlan OLAC Ltd. (Bicester, UK) or The Jackson Laboratory (Bar Harbor, ME), and were used between 6 and 26 wk of age.

Reagents

mAbs. The following anti–mouse antibodies were used (TIB refers to American Type Culture Collection [Bethesda, MD] designation): B21.2, rat IgG2b anti-IA^{b,d} (TIB 229), M1/70 (TIB 128), and 5C6 (reference 26); 10.2-16, mouse IgG2b anti-IA^{k,r,f,s} (TIB 93); and N418, hamster anti-CD11c (HB224).

Polyclonal Antisera. The following anti-IgG polyclonals were used at optimal concentrations (dilutions of 1:25–100): biotin sheep F(ab')$_2$ anti–mouse (B8899; Sigma, Poole, UK); rabbit anti–hamster (H1643; Sigma); and biotin-goat F(ab')$_2$ anti–hamster (107–066–142; The Jackson Laboratory).

Miscellaneous Conjugates. Texas red–streptavidin (TR-strp) (016–070–084; Jackson Immunoresearch Laboratories, West Grove, PA) was used at 1:1,000–2,000 dilution. PE-streptavidin (PE-strp) (7100–09; Southern Biotechnology Associates, Birmingham, AL) was used at 1:100–200 dilution.

Phagocytic Markers. Zymosan from *Saccharomyces cerevisiae* (Sigma) was prepared by boiling for 30 min in cation-free PBS followed by washing in PBS. *Staphylococcus aureus* and *Corynebacterium parvum* cultures (gifts from Public Health Laboratory, John Radcliffe Hospital, Oxford, UK) were fixed in 37% formaldehyde after washing in PBS. Carboxylated fluorescent latex beads (Fluoresbrite Carboxy YG 0.5-, 1-, and 3.5-μm diam; nos. 15700, 15702, and 17147) were from Polysciences Ltd. (Warrington PA). SRBC (Becton Dickinson, Abingdon, UK) were washed and used before or after opsonization for 30 min at 37°C with rabbit IgG anti-SRBC (diluted 1:200 Nordic Immunological Ltd., Maldenhead, UK), or with monoclonal IgM anti-SRBC (MAS 012b, diluted 1:2; Sera-Lab Ltd., Crawley Down, UK) plus fixed C3 as described (27).

Zymosan, bacteria, and SRBC were conjugated to FITC (Sigma) by incubating with fluorochrome overnight in 0.5 M NaHCO$_3$, pH 9.5, at 4°C or room temperature with constant shaking. Particles were washed in PBS and sterilized by irradiation from a ^{137}Cs

source before use. SRBC used for FITC conjugation were fixed for 30 min at 4°C on ice with 3.7% formaldehyde in PBS. Unconjugated and FITC-conjugated zymosan and formalin-fixed bacteria were stored at 4°C for up to 3 mo or at −20°C for longer periods. Fixed SRBC and FITC-SRBC were prepared fresh for each experiment.

For uptake studies, all particles were used at concentrations that were saturating for peritoneal macrophages (Mph) in similar assays.

Endocytic Markers and (Glycan) Inhibitors. FITC-BSA and man-nosylated FITC-BSA (FITC-ManBSA) were from Sigma. Purified soluble mannan and β-glucan were prepared and kindly provided by V. Kery (Washington University, St. Louis, MO) or were purchased from Sigma. Dextran T40 was from Pharmacia LKB Biotechnology (Piscataway, NJ).

Cell Preparations

Culture medium used throughout was RPMI 1640 supplemented with 10% heat-inactivated FCS (both from Gibco BRL, Paisley, UK), 2 mM glutamine (BDH, Merck Ltd., Atherstone, UK), 25 μM 2-ME (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK), 45 μg/ml penicillin, 45 μg/ml streptomycin, and 90 μg/ml kanamycin (antibiotics from Gibco BRL).

Epidermal Cells (EC) and LC. EC suspensions containing 0.5–2% LC were freshly prepared from mouse ear skin, or were cultured to obtain EC containing 1–4% LC, essentially as described (4). In some experiments, nonadherent EC cultured for at least 12 h were fractionated over dense BSA to obtain low-density cells (15–40% of the total population) containing 10–60% LC (4); phago-cytosis of particles did not noticeably affect LC density, and routinely <5% of LC were present in the high-density fraction.

Splenic DC. Spleen cell suspensions were fractionated over dense BSA, and DC (>70% purity) were obtained from the populations that detached after overnight culture of the low-density adherent cells (LODAC) as described (28). For studies with fresh splenic DC, the LODAC were harvested by vigorous washing with cold RPMI 1640 4–6 h after initial plating.

Peritoneal Mph. Adherent Mph were obtained from peritoneal cells lavaged from normal mice or from mice injected intraperitoneally 4 d previously with 1 ml thioglycollate broth (a gift from R. da Silva, Sir William Dunn School of Pathology, Oxford, UK).

Immunofluorescent Cell Labeling

Labeling of Attached EC. EC were incubated in complete culture medium with particles for the indicated times, and fractionated over BSA. The cells were washed and plated for 15–30 min at 37°C on coverslips coated with poly-L-lysine (PLL; Sigma), as per manufacturer's instructions. The attached cells were fixed in 3.7% formaldehyde in PBS for 15 min and stained for MHC class II with biotinylated-TIB 229 (kindly prepared and made available by J. A. Roake, University of Oxford) followed by PE-strp or TR-strp; antibody was omitted for control samples. Each incubation was for 40–50 min, and antibodies and conjugates were diluted in PBS containing 0.02% sodium azide and 1% heat-inactivated FCS (washing solution; WS), which was also used for washing between each stage. Coverslips were washed in distilled water and mounted in Aquamount (BDH, Merck Ltd.). Stained cells were visualized and photographed on an Axioskop microscope equipped with UV illumination and FITC and Texas red filters (Carl Zeiss, Inc., Thornwood, NY).

Labeling of Cell Suspensions for FACS® Analysis. Cell suspensions were fixed with 3.7% glutaraldehyde for 15 min on ice and

washed in PBS. Subsequent incubations with antibodies and fluorescent conjugates (diluted in WS) were for 40–60 min on ice, washes were in WS, and cells were stored at 4°C in 1% formaldehyde in WS until analysis.

LC from C57BL/6 and BALB/c mice in fixed EC suspensions were stained with biotin-TIB 229 followed by PE-strp. LC from C3H/HeN mice were stained with TIB 93, or no antibody as a negative control, followed by biotin sheep F(ab')₂ anti–mouse IgG and PE-strp.

DC-enriched spleen cell suspensions were preblocked with heat-inactivated normal mouse serum (diluted 1:25; Jackson Immunoresearch) and stained with HB224 (N418; a gift from R. M. Steinman, The Rockefeller University, New York, NY), or no antibody as a negative control, followed by biotin goat F(ab')₂ anti–hamster IgG and PE-strp.

Phagocytic and Endocytic FACS® Assays

Cell suspensions were incubated ("pulsed") with fluorescent particles on tissue culture dishes (Falcon Labware, Oxnard, CA) for the indicated periods of time, and nonadherent cells were harvested by washing the surface of the dish with ice-cold RPMI 1640. For some phagocytosis inhibition studies, cells were harvested before the assay and preincubated in polypropylene tubes (Falcon Labware) for 30 min with inhibitors before pulsing with particles in the same tubes in the continuous presence of inhibitors; similar results were obtained with assays performed in either tubes or culture dishes. For uptake studies of soluble fluorescent tracers, cells were generally pulsed in tubes.

After staining of LC or DC with the appropriate antibodies in conjunction with PE, samples were analyzed by flow cytometry on a FACScan® (Becton Dickinson & Co., Mountain View, CA) using Consort 30 software. The instrument was calibrated for each experiment using double-negative and single-positive samples. After gating out debris and free particles, live gates were set around the brightest PE⁺ population. Up to 5,000 LC or DC were acquired and analyzed for FITC fluorescence. Data were collected using a logarithmic recorder but mean channel fluorescence values were converted to a linear scale. Essentially all TIB 229⁺ cells in EC suspensions also expressed NLDC-145 (29).

Electron Microscopy

For transmission electron microscopy of LC-enriched EC suspensions (Fig. 1), cells were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate/1% sucrose buffer (60 min total). After washing in the same buffer, pellets were postfixed in 2% osmium tetroxide (50 min), embedded in 1% agar, dehydrated through a graded acetone series, and embedded in Emix resin (Agar Scientific, Stansted, UK). Thin sections were cut on a Reichert-Jung ultramicrotome, stained with lead citrate, and visualized and photographed in an electron microscope operating at 80 kV (100CX; Jeol Ltd., Tokyo, Japan).

For immunoelectron microscopy of DC-enriched spleen cell suspensions (Fig. 4), cells were processed as described (30). Cryosections were cut at −90°C with a Reichert-Jung Ultra Cut ultramicrotome equipped with a FC4E cryosystem (Leica Inc., Deerfield, IL) and were stained with N418 followed by rabbit anti–hamster IgG and a protein A–10 nm gold conjugate prepared as described (31). Staining was as described (30) except that PBS containing 10% FCS was substituted for 0.1% BSA-PBS. Specimens were viewed and photographed in an electron microscope (10A; Carl Zeiss, Inc.).

Results

Initial studies were performed with cells from C57BL/6 mice and later extended to other strains; the data relate to the former unless stated. Because of the difficulty in obtaining sufficient numbers of cells, replicate samples could not always be assayed in the same experiment, but all experiments were repeated multiple (3 to >20) times with identical results unless otherwise noted.

Freshly Isolated LC Are Phagocytic In Vitro. Light microscopic observations suggested that fresh LC could internalize latex microspheres and zymosan particles. Fresh EC suspensions were incubated with particles, and LC-enriched (BSA) fractions were examined by transmission EM (Fig. 1). LC were identified by cytology and distinguished from keratinocytes, which, for example, possess a distinct rim of peripheral intermediate filaments (not shown). Internalization of 0.5–3.5-μm latex microspheres was observed (Fig. 1, *A–C*) within membrane-bound phagosomes (Fig. 1 *D*). Internalized zymosan particles assumed an irregular shape with a distorted electron-dense core (Fig. 1 *E*), in contrast to the oval appearance of extracellular particles (not shown), presumably as a result of intracellular degradation.

Fresh EC were then pulsed with fluoresceinated particles and examined by two-color UV microscopy (e.g., Fig. 2, and data not shown). Most MHC class II–positive cells (LC) were associated with latex beads and zymosan. To confirm that FITC-zymosan had been internalized, viable cells were labeled with Texas red–conjugated anti-FITC antibodies after pulsing; ∼95% of cell-associated particles fluoresced green only (data not shown). LC typically internalized two to eight zymosan particles during overnight culture, as well as intact *S. cerevisiae*, whereas stimulated Mph phagocytosed >15 particles in 10 min. Uptake of FITC-labeled *C. parvum* and *S. aureus* by LC (and by a subset of MHC class II–negative keratinocytes, as reported in reference 32) was also visualized (e.g., Fig. 2).

To determine whether fresh LC can phagocytose particles via Fc receptors for IgG (CDw32) or via complement receptors (CD11b/18), LC were pulsed with opsonized SRBC (see Materials and Methods). However, unlike control Mph, no binding or uptake of SRBC by fresh LC was observed, and as a further difference LC did not internalize colloidal carbon (not shown). Various particles were also preincubated with fresh mouse serum before addition to EC cultures. There was no uptake of mouse erythrocytes incubated in the presence or absence of mouse serum. Preincubation had a minimal effect on the uptake of zymosan or *S. aureus* (not shown), but the number of LC that internalized *C. parvum* was increased from ∼50–100% (by FACS® analysis; see below).

Cultured LC and Splenic DC Have Little Phagocytic Capacity. By UV microscopy, the capacity of LC to phagocytose latex beads, zymosan, *C. parvum*, and *S. aureus* was greatly reduced after culture (e.g., Fig. 2). The extent of phagocytosis by fresh and cultured LC was then quantified by two-color FACS® analysis (examples are in Fig. 3). The markedly decreased phagocytic capacity of cultured vs. fresh LC is evi-
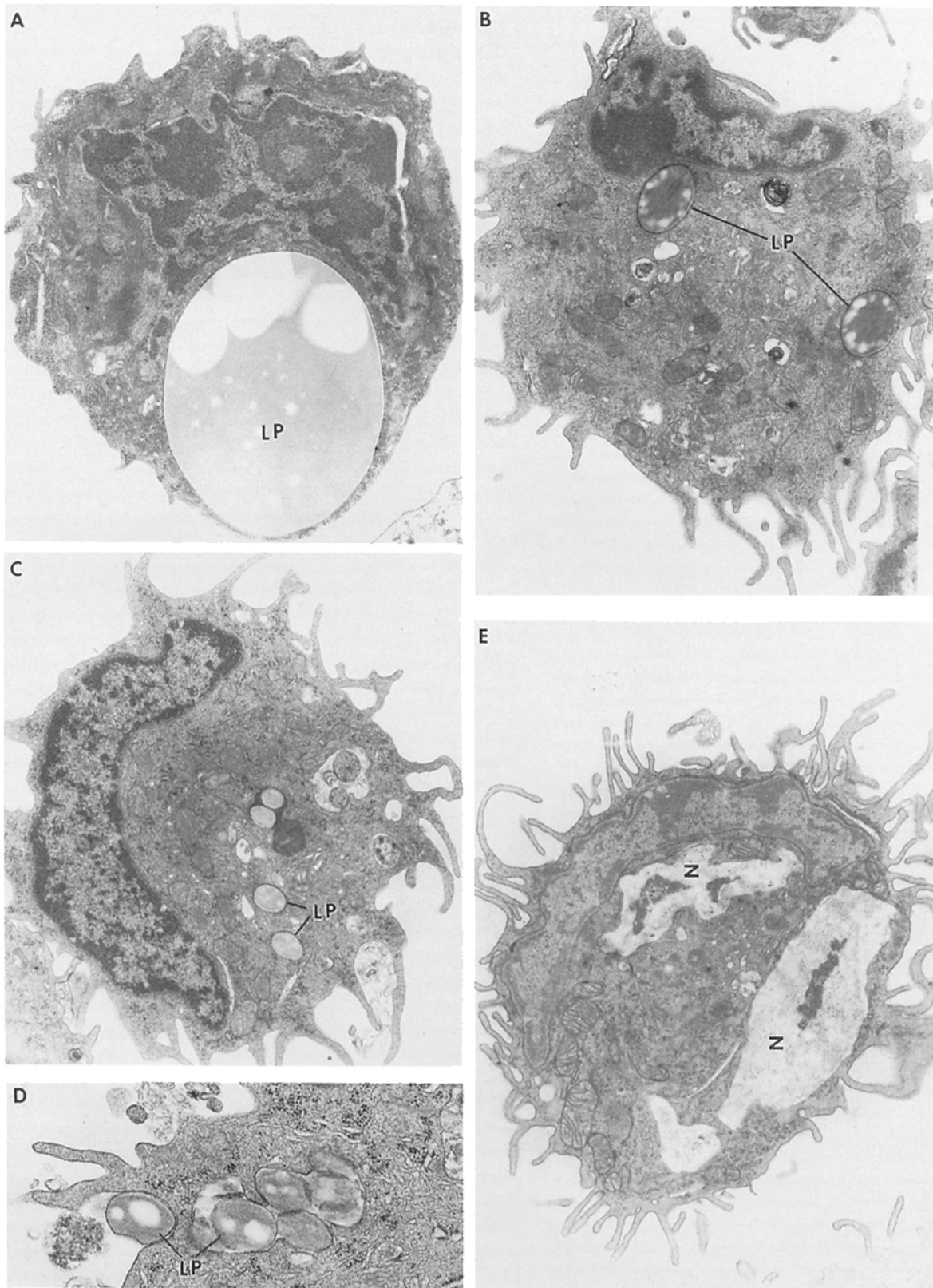
Figure 1. Transmission electron microscopy. Phagocytosis of latex microspheres and zymosan by freshly isolated LC. Fresh EC were incubated with particles for 12–18 h and low-density cells were processed for transmission electron microscopy. Particles: (*A*) 3.5-, (*B*) 1-, (*C* and *D*) 0.5-μm latex microspheres (*LP*); (*E*) zymosan (*Z*). (*A*) ×16,000, (*B* and *C*) ×13,000, (*D*) ×24,000, (*E*) ×12,000 (original magnifications). Although not shown here, Birbeck granules were occasionally observed in cells of similar morphology.
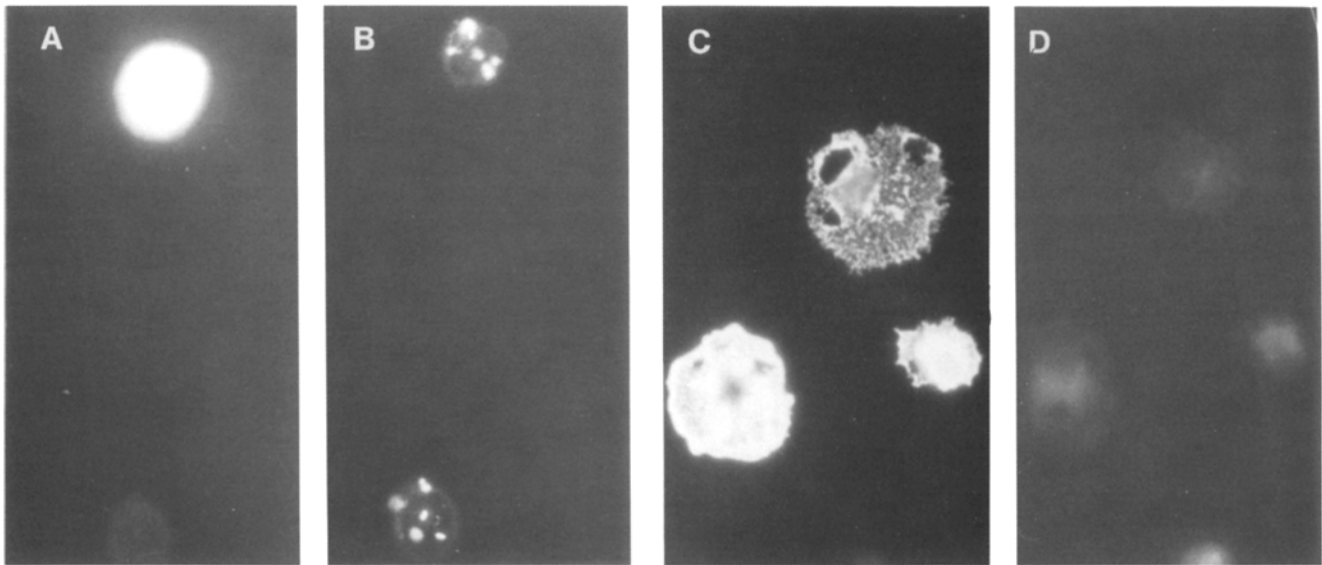
Figure 2. UV microscopy. Freshly isolated LC phagocytose *C. parvum*, but their phagocytic capacity is markedly reduced after culture. Fresh (*A* and *B*) or 72-h cultured EC (*C* and *D*) were incubated with FITC-conjugated, fixed *C. parvum* for 18 h, and low-density cells attached to PLL coverslips were labeled with TIB 229 (anti–MHC class II) in conjunction with Texas red (TR) conjugates. For each pair of UV photographs, the left side is TR fluorescence and the right side is FITC fluorescence of the same field; ×1,000. Similar results were obtained for other particles (see text).

dent from the percentage of cells associated with particles and the mean fluorescence values (Fig. 3, *c*, *e*, and *g*), and from comparison of their phagocytic indices (Fig. 3, *d*, *f*, and *h*). LC retain zymosan for at least 4 d (Fig. 3 *g*; other particles not examined), but a reduction in the percentage of cells associated with particles and the mean fluorescence indicates that some antigen is lost, possibly due to degradation (compare with Fig. 1 *E*).

The observation that some cultured LC retain phagocytic activity (e.g., Fig. 3, *c–h*) led us to re-examine splenic DC using the FACS® assay. DC-enriched spleen cell suspensions were incubated with zymosan or latex microspheres before (day 0) or after (day 1) culture, and DC were identified by staining with HB224 (N418; reference 33). The percentage of day 0 cells associated with particles in different experiments was 15–35% for zymosan and 35–50% for latex, but in both cases was reduced to 5–15% on day 1 (data not shown). Phagocytosis by freshly isolated splenic DC was confirmed by immunoelectron microscopy (Fig. 4, *A* and *B*). Uptake by DC was more limited than by contaminating N418-negative cells, presumably Mph (Fig. 4 *C*).

We conclude that the phagocytic capacity of LC is markedly reduced after maturation in culture (a summary is in Table 1). The phagocytic capacity of freshly isolated splenic DC is also reduced after overnight culture, presumably due to maturation of a subset of cells (14, 34–36).

Receptors Mediating Uptake of Zymosan by Fresh LC. Quantitation of zymosan uptake by LC from different strains of mice revealed differences in both phagocytic activity and the kinetics with which this decreased during maturation. With long (12–18-h) incubations, phagocytosis by fresh LC from C57BL/6 and BALB/c mice was comparable for most parti-

cles tested (data not shown). The only significant differences were noted for zymosan: >95% of C57BL/6 LC phagocytosed this particle compared with 50–70% of BALB/c LC (levels were intermediate for C3H/He LC) and mean fluorescence values differed correspondingly (not shown). With short (90-min) incubations, C57BL/6 LC exhibited a reproducible rise in their phagocytic index for zymosan (but not for 1-μm latex beads) from 0 to 6 h of culture, followed by a decline, whereas this initial increase did not occur for BALB/c LC (Fig. 5, *a* and *b*). Binding at 4°C showed a similar pattern, suggesting that these kinetics are due to strain differences in expression of receptors for zymosan.

At least three receptors are involved in phagocytosis of zymosan by monocytes and/or Mph, namely the mannose receptor (MR; also known as the mannosyl-fucosyl receptor; 37, 38), the β-glucan receptor (39, 40), and complement receptor type 3 (CR3; CD11b/18; 41, 42), which can be inhibited by mannans, β-glucans, and anti-CR3 antibodies, respectively. Uptake of zymosan (but not latex beads) by LC was inhibited by both mannan and β-glucan but not by dextran (Fig. 5, *c* and *d*). Therefore, glycan receptors may be expressed by fresh LC but the different plateau levels for inhibition of zymosan uptake by C57BL/6 LC (75–80%; Fig. 5 *c*) compared with BALB/c LC (40–50%) suggests they are differentially expressed in a strain-dependent manner. CR3 receptors do not appear to be involved since no inhibition of zymosan uptake by LC from either strain of mice was observed when anti-CR3 antibodies (M1/70 [41] or 5C6 [26]) were included in 16-h pulses at 10–200 μg/ml; antibody binding to resident peritoneal Mph was saturating at 1–5 μg/ml (data not shown).

Uptake of FITC-conjugated mannosylated-BSA was also
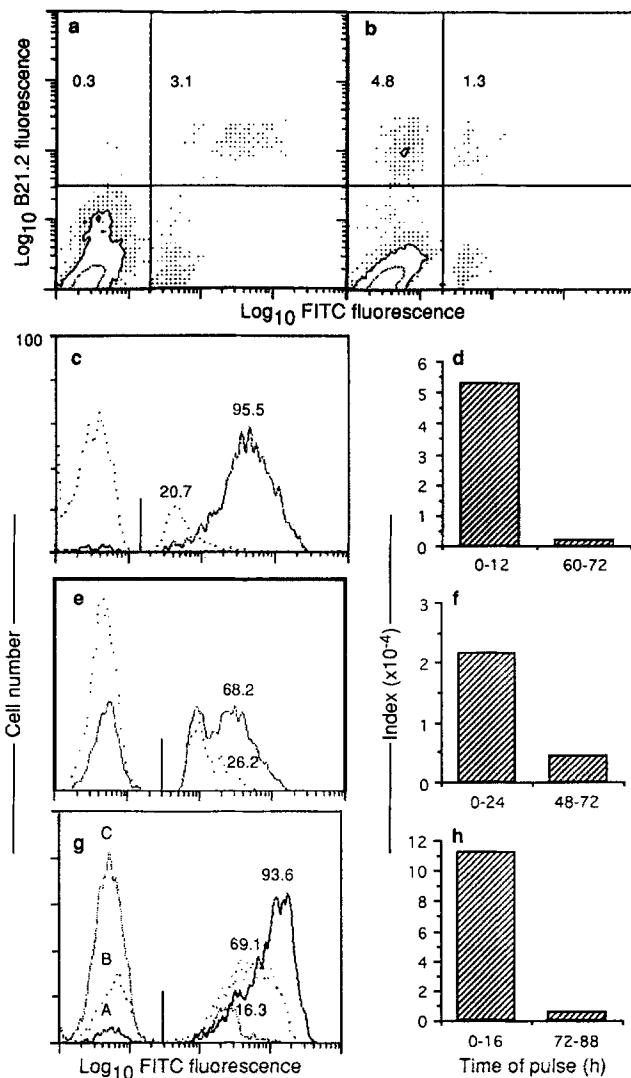
Figure 3. FACS® analyses. Freshly isolated LC phagocytose *S. aureus*, latex microspheres, and zymosan, but their phagocytic capacity is markedly reduced after culture. Fresh or cultured EC were incubated with fluorescent (FITC) particles and bulk populations were labeled with TIB 229 (anti–MHC class II) in conjunction with PE, and analyzed by two-color flow cytometry. (*a* and *b*), double fluorescence plots of fresh (*a*) and 60-h cultured EC (*b*) pulsed with FITC–*S. aureus* for 12 h, the numbers indicating percentage of cells in that quadrant; (*c*) FITC fluorescence of gated B21-2⁺ cells from *a* (fresh EC, *solid line*) and *b* (cultured EC, *dotted line*), the numbers indicating the percentage of FITC⁺ LC for each population; (*d*) data from *c* expressed as an index, the product of percentage FITC⁺ LC and mean fluorescence values; (*e* and *f*) uptake of 1-μm fluorescent latex beads (represented as in *c* and *d*) by fresh and 48-h cultured EC after a 24-h pulse; (*g* and *h*) uptake of FITC-zymosan (represented as in *c* and *d*) by LC pulsed from 0 to 16 h (*g*, *solid line*), pulsed from 0 to 16 h and recultured until 88 h (*g*, *widely spaced dotted line*), or pulsed from 72 to 88 h (*g*, *closely spaced dotted line*).

Table 1. *Summary of Studies on Phagocytosis by LC*

Particle*	Fresh LC	Cultured LC	Mph
Zymosan	+ + +	+	+ + + + +
Zymosan + nms	+ + +	ND	(+ + + + +)
S. cerevisiae	+ + +	ND	(+ + + + +)
0.5-μm latex beads	+ + +	±	+ + + + +
1-μm latex beads	+ +	±	+ + + + +
3.5-μm latex beads	+	±	+ + + + +
C. parvum	+ +	±	+ + + +
C. parvum + nms	+ + +	ND	(+ + + + +)
S. aureus	+ + +	+	+ + + +
S. aureus + nms	+ + +	ND	(+ + + + +)
Colloidal carbon	−	ND	(+ + + + +)
SRBC ± IgG	−	ND	+ + +
SRBC ± IgM	−	ND	−
SRBC ± complement	−	ND	±‡
Aldehyde-fixed SRBC	−	ND	+ + + + +
FITC-SRBC	−	ND	+ + + + +
Mouse RBC ± nms	−	ND	±

A compilation of results from different experiments. The plus and minus signs are used solely for comparison between data for different particles from microscopic observations and flow cytometry; symbols in parentheses reflect published data. nms, normal mouse serum; RBC, erythrocytes; SRBC, sheep RBC.

* Uptake of zymosan, *C. parvum*, and *S. aureus* was similar whether or not the particles were conjugated to FITC. For latex, differences in mass between beads of different sizes make direct comparisons of uptake difficult. ‡Particles were attached to Mph but not to LC.

kinetics were observed in the presence of competing ligand, and for uptake of FITC-BSA and Lucifer yellow (not shown). Therefore, during maturation in culture, LC may downregulate both their endocytic (pinocytic) capacity as well as their ability to phagocytose particles (e.g., Table 1).

The relationship between the mannose binding activity expressed by fresh LC and the Mph MR was investigated in preliminary experiments (data not shown). A rabbit polyclonal antiserum was raised against MR purified from the J774E cell line (44). This reagent did not inhibit uptake of zymosan, FITC-mannose-BSA, or FITC-mannan by Mph, but it identified a 165–180-kD component by immunoprecipitation and Western blotting of Mph lysates. However, no component was detectable in Western blots of lysates from LC-enriched or LC-depleted EC cultured for 6 h (when uptake of zymosan and FITC-mannose-BSA is maximal; see Fig. 5 *a*) or for 3 d, even when at least an order of magnitude more LC than Mph protein was used and despite the ability to detect MHC class II molecules in these preparations. This suggests either that the amount of protein present in LC is considerably less than that in Mph, or that a distinct receptor is responsible for the mannose-binding activity of fresh LC.

examined, a well-characterized soluble ligand that is endocytosed via MR in Mph (43). The kinetics of uptake by C57BL/6 LC were similar to those of FITC-zymosan (data not shown; see Fig. 5 *a*). However, the overall pinocytic capacity of the cells appeared to change in a similar fashion, since the same
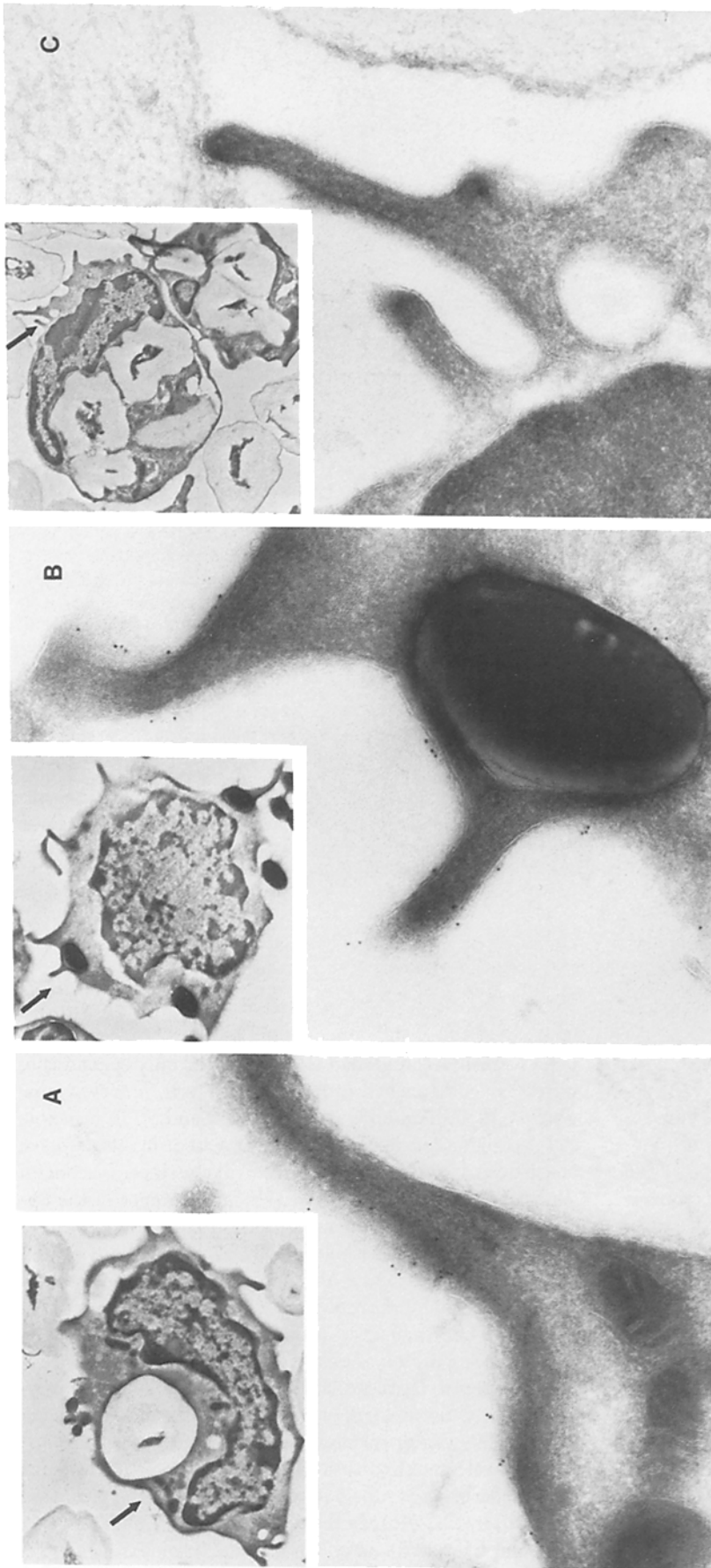
Figure 4. Transmission immunoelectron microscopy. Phagocytosis of zymosan and latex microspheres by freshly isolated splenic DC. Low-density spleen adherent cells were harvested 4–6 h after initial plating, incubated with particles, and processed for immunoelectron microscopy with N418 and 10-nm gold (see Materials and Methods). The area indicated on the insets (×5,100) is enlarged on the main photographs (×49,000). (*A* and *B*) Uptake of zymosan (*A*) and 1-μm latex (*B*) by N418+ DC; (*C*) uptake of zymosan by N418− macrophages, photographed on the same grid as *A*, as an internal control for specificity of staining (similar results were obtained after omission of N418, not shown).
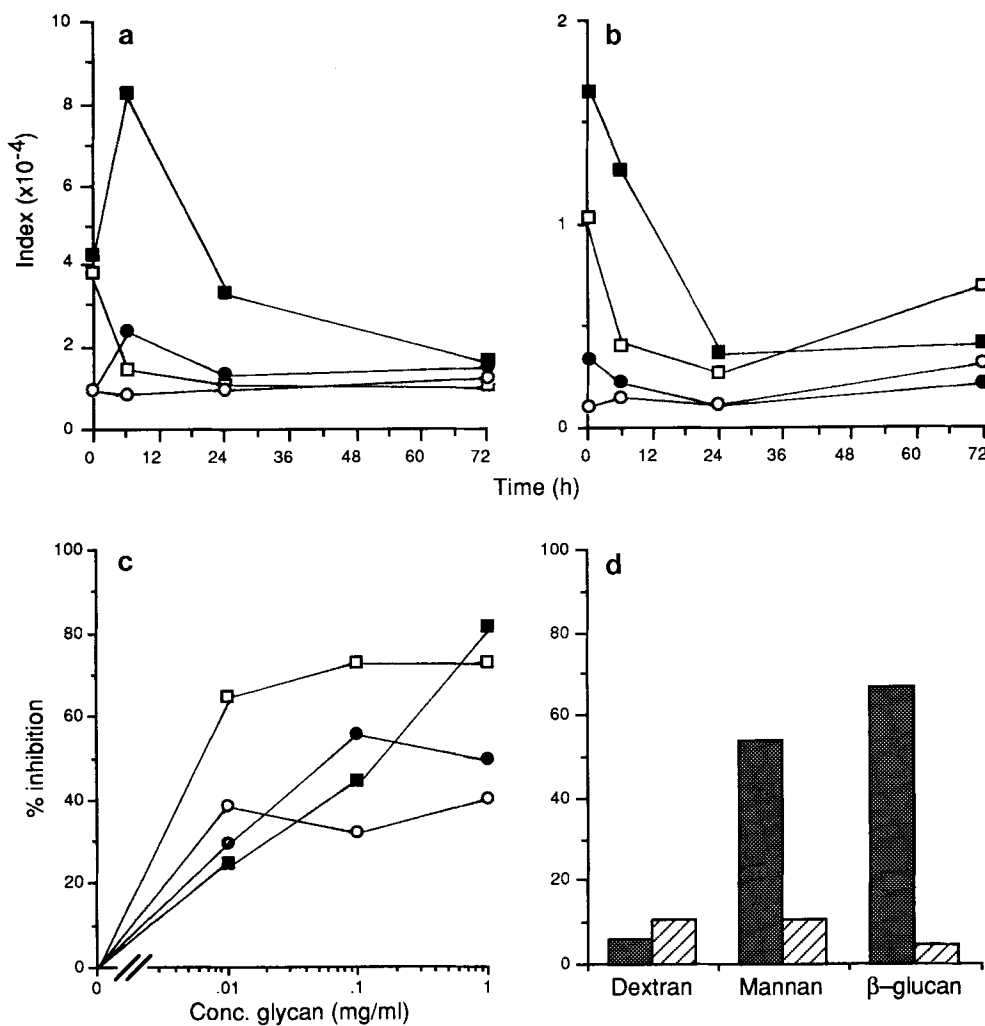
Figure 5. FACS® analyses. Kinetics of uptake or binding of zymosan or latex microspheres by LC, and effect of glycans. (*a* and *b*) EC from C57BL/6 (*filled symbols*) and BALB/c mice (*open symbols*) were pulsed at the indicated times of culture for 90 min with FITC-zymosan (*a*) or 1-μm fluorescent latex beads (*b*) at either 37°C (*squares*) or on ice (*circles*), and uptake or binding by LC was assessed by flow cytometry (see Fig. 3). (*c*) EC from C57BL/6 (*squares*) and BALB/c mice (*circles*) were cultured for 6 h, preincubated with the indicated concentrations of purified mannan (*open symbols*) or β-glucan (*filled symbols*), and then pulsed with FITC-zymosan for 90 min at 37°C in the continuous presence of glycan. (*d*) Freshly isolated C57BL/6 EC were incubated for 16 h at 37°C with saturating concentrations of FITC-zymosan (*shaded bars*) or 1-μm fluorescent latex beads (*hatched bars*) in the presence or absence of the indicated glycans at 1 mg/ml. For *c* and *d*, results from FACS® analysis (see Fig. 3) are expressed as index converted to percentage inhibition relative to controls incubated in the absence of glycans (in which >95% of LC were associated with the respective particles).

Discussion

In this report we have demonstrated that freshly isolated LC can phagocytose a variety of particles ranging in size from 0.5 to 6 μm; the cutoff for fluid phase pinocytosis is conventionally taken to be ~0.5 μm (45). Internalization of (4–6-μm) zymosan particles was visualized by EM (Fig. 1) and their intracellular localization was confirmed from experiments in which FITC-zymosan was shown to be inaccessible to an anti-FITC reagent (see Results). Internalized zymosan assumed a markedly different structure compared with extracellular particles (Fig. 1 D) presumably due to intracellular degradation, as also suggested by FACS® analysis (Fig. 3 g). Uptake of 1–3.5-μm latex beads, apparently within phagosomes of LC, was observed (Fig. 1, A–D). Both types of particle were phagocytosed by a subset of freshly isolated splenic DC (Fig. 4), conceivably because some of these cells are immature (34–36). Maturational heterogeneity could also explain why some VC of afferent lymph can internalize latex beads (24).

UV microscopy of cells pulsed with fluorescent particles (e.g., for FACS® analysis) further established that most par-

ticles were internalized by LC at 37°C, and only occasionally were they cell attached (not shown); it was, however, possible to detect binding at 4°C (Fig. 5, *a* and *b*). In principle FITC-conjugation might have potentiated or induced uptake of particles but this does not seem likely since, in control studies (data not shown), there was no difference in the uptake of FITC-conjugated and unmodified zymosan, and no phagocytosis of FITC-labeled SRBC. Phase and differential interference microscopy also allowed clear distinction between most bound and internalized particles, and was used to confirm uptake by LC in all cases (not shown).

Microscopic observations (e.g., Fig. 2) and FACS® analyses (Fig. 3, *a–d*) revealed that freshly isolated LC can phagocytose both *S. aureus* (diameter, 0.7–1.2 μm) and *C. parvum* (length, 2–6 μm; diameter, 0.5–1 μm). The former is of immunological interest because of the production of enterotoxins that can function as superantigens, and that can be presented to T cells by DC (46). In relation to the latter, the cell walls of the corynebacteria are chemically and serologically related

to those of the mycobacteria (and nocardiae), and it is of interest that Poulter et al. (47) described rare CD1^{+} cells in the dermis of leprous skin that contained *M. leprae*; where these cells acquired the organisms remains to be established.

There were clear quantitative and qualitative differences in the phagocytic capacity of LC compared with Mph: LC internalized a smaller number of particles over a longer period of time, and fresh LC did not attach or phagocytose IgG- or complement-coated SRBC (the former in contrast to a previous report; 19). This was not simply due to the large size of SRBC since zymosan is of a similar size, and erythrocytes are present in LC migrating from human skin explants (Larsen, C. P., and J. M. Austyn, unpublished observation) and in VC from afferent lymph (22). However, uptake of *C. parvum* by LC was markedly increased after incubation with mouse serum, perhaps due to activation of complement or binding of natural antibodies. This observation could suggest that phagocytosis via Fc and/or complement receptors by LC requires coligation of other receptors, as described for Mph (e.g., reference 45), and might explain why erythrocytes have been observed within LC and VC, but are not phagocytosed in vitro (see Introduction). Uptake of soluble immune complexes by LC has been inferred from previous studies (16, 22) but it is not clear whether internalization was mediated by Fc and/or complement or other receptors.

Uptake of zymosan by fresh LC is mediated in part by a glycan receptor(s) that may be related to MR of Mph. These receptors may be differentially expressed by LC from different strains of mice, and appear to be downregulated during LC maturation. Strain-dependent expression is indicated by the different plateau levels of glycan inhibition for C57BL/6 and BALB/c LC (Fig. 5 c), and the differential kinetics of zymosan uptake during maturation that are mirrored by binding at 4°C (Fig. 5 a). The apparent increase in uptake by C57BL/6 LC at 6 h (Fig. 5 a) could be due to recovery from trypsin used to isolate the cells, but it is clearly different for LC from the two strains of mice. Decreased expression during maturation (e.g., from 0 to 72 h) is indicated by the kinetics of zymosan binding and uptake (Fig. 5 a), and by uptake of FITC-mannose-BSA, which shows a similar pattern (not shown). However, the observation that pinocytic activity changes in a similar fashion complicates the picture (see Results). For example, the MR of Mph recycles through the early endocytic pathway and, at steady state, 80–90% is intracellular (48, 49). Changes in the pinocytic rate could affect this distribution, making more or less receptor available for binding, and leading to specific changes in uptake without regulation of the total receptor expression per se. Whatever the case, the availability of mannose-type receptors to exogenous ligands is decreased during maturation of LC.

We have recently shown that DC isolated from mouse hearts and kidneys can also phagocytose zymosan (J. M. Austyn et al., manuscript submitted for publication). It would make sense for a cell that ultimately initiates immune responses to have broad-spectrum recognition systems for microorganisms. In this regard, a role for MR in host defense has been suggested since bacteria and yeasts cannot process high mannose-type chains, but express a variety of mannans and glucans on their surfaces, and the composition of cell walls of different yeasts is remarkably similar. It is also noteworthy that MR has been implicated in attachment and infection of Mph by *Leishmania* (50, 51), and that LC can internalize and present antigens from these organisms (52, 53); it would be interesting to establish whether strain differences in resistance or susceptibility to infection by *Leishmania*, i.e., induction of Th1 or Th2 responses, are related to differences in phagocytosis by their LC. Since phagocytic activity can be conferred by particular receptors (54–56), downregulation of mannose-type receptors by LC may contribute directly to the marked reduction in their phagocytic activity during maturation.

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