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Langerhans cells transport *Leishmania major* from the infected skin to the draining lymph node for presentation to antigen-specific T cells*

Murine epidermal Langerhans cells (LC) have been shown to internalize *Leishmania major*, a cause of human cutaneous leishmaniasis, and to stimulate a vigorous parasite-specific T cell response. The present study emphasizes the critical role of LC in leishmaniasis by documenting directly that LC have the ability to transport *L. major* from the skin to the draining lymph node (LN). This was revealed by irreversible labeling of LC with a fluorescent cell linker and *in vivo* tracking. In contrast, no migration to the LN was seen with *L. major*-infected macrophages. These findings were consistent with the results of mixed labeling immunohistology showing that early in infection the expression of parasite antigen in the LN draining the lesion was confined to dendritic cells and could not be detected in macrophages. Furthermore, dendritic cells in LN draining the site of cutaneous infection stimulated *L. major*-primed T cells *in vitro* and, most notably, were able to activate unprimed T cells capable of mediating parasite-specific delayed-type hypersensitivity reactivity *in vivo*. Taken together, the results indicate that LC capture *L. major* in the skin and transport it to the regional LN for initiation of the specific T cell immune response.

1 Introduction

The spectrum of human disease patterns caused by protozoan parasites of the genus *Leishmania* can be reproduced by experimental infection of mice with *Leishmania major*, a cause of human cutaneous leishmaniasis. A considerable body of evidence demonstrates the importance of T cell-dependent immunity in both resistance and susceptibility to disease [1]. In the mammalian host, the parasites exist as obligatory intracellular amastigotes. We have recently shown that, in addition to MΦ, epidermal Langerhans cells (LC) are able to phagocytose *L. major* and serve as host cells for the parasite both *in vitro* and *in vivo* in the infected skin [2]. Furthermore, LC are highly active in inducing the proliferation and lymphokine production of *L. major*-specific T cells *in vitro* [3]. These findings suggest that LC are critical APC in cutaneous leishmaniasis.

LC are members of the dendritic cell lineage that forms a system of potent APC in lymphoid and nonlymphoid tissues [4–7]. Dendritic cells constitutively express high levels of MHC class II molecules and possess the unique capacity to induce primary T cell responses. Thus, they are clearly distinct from cells of the MΦ/monocyte lineage. Moreover, evidence is accumulating that, upon stimulation by alloantigens and contact allergens, dendritic cells in peripheral nonlymphoid organs such as epidermal LC are able to migrate via the lymphatics into the T cell areas of lymphoid tissues [8–10]. This translocation provides an efficient means of transporting Ag from the site of first encounter in the skin to the draining LN for initiation of the T cell immune response.

The possibility exists that the above concept also accounts for the effective immunogenic presentation of Ag from microorganisms infecting the skin. This aspect has not yet been analyzed. In the present study, we examined the capacity of murine epidermal LC to transport *L. major* Ag from the skin to lymphoid tissues for presentation to T cells. Our data provide direct evidence that *L. major*-infected LC migrate to the regional LN and stimulate an Ag-specific T cell immune response.

2 Materials and methods

2.1 Mice

Female mice of the inbred strain BALB/c and athymic BALB/c nu/nu (nude) mice were 5 to 7 weeks of age at the onset of experiments. All mice were purchased from Charles River Breeding Laboratories (Sulzfeld, FRG) and, during experimentation, were maintained under conventional conditions in an isolation facility.

2.2 Parasites and infection of mice

The origin and propagation of the *L. major* isolate have been described elsewhere [11]. The cloned virulent line used for this study was confirmed to be *L. major* by isoenzyme analysis and was maintained by passage in BALB/c mice. Promastigotes were grown *in vitro* in blood agar cultures. Stationary-phase promastigotes were washed in PBS and, for i.d. infection of mice, 1×10^5 to 20×10^6 organisms were injected in a volume of 10 µl on the dorsum, using a 100-µl Hamilton syringe mounted with a 30-gauge Yale needle. Amastigote suspensions were prepared from skin lesions 2 to 3 weeks later as described [12]. For the

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Abbreviations: **LC:** Langerhans cells **RT:** Room temperature

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preparation of *L. major* lysate, stationary-phase promastigotes were subjected to three cycles of rapid freezing and thawing.

2.3 Culture medium and Ab

Click's RPMI 1640 medium (Gibco Laboratories, Eggenstein, FRG) was supplemented with 10% FCS, 2 mM L-glutamine, 10 mM Hepes buffer, 100 μg/ml penicillin, 160 μg/ml gentamycin (all these supplements purchased from Seromed-Biochrom; Berlin, FRG), 7.5% $NaHCO_3$ and 5×10^{-5} M 2-ME.

Rat mAb directed against MHC class II Ag I-A (b,d,q haplotypes) and I-E (d,k haplotypes), from hybridoma M5/114.15.2 [13], were used as culture SN. Rat mAb against nonlymphoid dendritic cells, from hybridoma NLDC-145 (Dianova, Hamburg, FRG; [14]), against Mac-1, from hybridoma M1/70 (Boehringer Mannheim, Mannheim, FRG; *[15]) and* rat mAb F4/80 [16], directed against mouse MΦ, as well as mouse mAb WIC79.3 [17, 18], recognizing a polymeric epitope specific for the surface lipophosphoglycan of *L. major,* were used as purified protein. Polyclonal Ab to *L. major* were raised in rabbits by s.c. and i.m. injections of promastigotes in CFA followed by several boosters of promastigotes in IFA and collection of the serum. A FITC-conjugated goat anti-rat Ig Ab (Medac, Hamburg, FRG) served as second-stage reagent for fluorescence staining. Biotin-conjugated mouse anti-rat Ig Ab (Dianova) and gold-conjugated goat anti-rabbit Ig Ab (Dako, Hamburg, FRG) were used as second-stage reagents for immunohistochemical staining.

2.4 Preparation of MΦ and epidermal cells

For the preparation of MΦ, thioglycolate-elicited PEC were washed, resuspended in culture medium and were allowed to adhere for 4 h at 37 °C, in a humidified atmosphere of 5% CO_2. Thereafter, nonadherent cells were removed by extensive washing with culture medium. Single-cell suspensions of epidermal cells were prepared from mouse ear skin by trypsinization procedures as described [19]. A concentration of 1% trypsin (Flow Laboratories, Meckenheim, FRG) was used for processing the ventral, thick ear halves (90 min), and 0.6% trypsin for the dorsal, thin ear halves (45 min). These preparations contained 3 to 5% MHC class II-bearing LC and were absolutely devoid of MΦ [19], as documented by the lack of staining with mAb F4/80 after 24 h of culture (fluorescence labeling).

2.5 T cells

For the generation of Ag-primed T cells, mice were immunized s.c. at the base of the tail and into the hind footpads with *L. major* lysate (equivalent to 70×10^6 parasites) in CFA. After 8 days, inguinal and popliteal LN cells were collected and were restimulated with Ag *in vitro* in bulk cultures [20]. This procedure reduces the syngeneic MLR that occurs in the absence of Ag when dendritic cells are co-cultured with primary T cells [21]. In 16-mm macrowells, 2.5×10^6 LN cells from *L. major*-immunized mice were restimulated with parasite lysate (equivalent to 2×10^6 organisms) in culture medium supplemented with 10% FCS for 10 days. Thereafter, viable cells were isolated by floatation on Ficoll-Paque columns (1.077 g/l; Pharmacia, Freiburg, FRG) and were used as responder cells at a dose of 1×10^5 per microwell.

The *L. major*-specific $CD4^+$ T cell clone L1/1 (kindly provided by Dr. M. Lohoff) was established from parasite-infected BALB/c mice by LD techniques and sequential stimulation with *L. major* lysate and lymphokine-containing culture SN as described [22]. Upon specific stimulation, L1/1 cells secrete IL-3, IL-4, IL-5 and IL-6, but not IL-2 or IFN-γ, thus showing the characteristics of Th2 type cells. Before use as responder cells at a dose of 2×10^4 per microculture well, L1/1 cells were rested for more than 14 days after the last restimulation with *L. major* lysate and irradiated spleen cells.

2.6 Assay for proliferation of T cells

T cells from *L. major*-primed mice or cloned *L. major*-specific T cells were cultured with varying numbers of APC in the absence of exogenous Ag. As the source of APC, irradiated (900 rad) single-cell suspensions of LN draining the site of i.d. injection of *L. major* promastigotes or *L. major* lysate were used. For depletion of dendritic cells, the LN cells were incubated with mAb NLDC-145 for 30 min on ice followed by removal of mAb-coated cells with anti-rat IgG coupled to magnetic polystyrene beads (Dynabeads M-450; Dynal, Hamburg, FRG) using a magnet. After this treatment, the cell suspension did not contain detectable numbers of $NLDC\text{-}145^+$ cells, as documented by fluorescence labeling. Culture was performed in 96-well flat-bottom microtiter plates (Nunc, Wiesbaden, FRG) in a final volume of 0.2 ml of medium supplemented with 0.5% heat-inactivated mouse serum or 10% FCS. After 48 to 60 h, [^{3}H] dThd (0.5 μCi/well; Du Pont De Nemours, Dreieich, FRG) was added for the final 18 h of culture. The cells were harvested onto filter strips using a semi-automated cell harvester and incorporation of [^{3}H] dThd was measured in a liquid-scintillation counter. Data were expressed as the arithmetic mean cpm ± SD of triplicate cultures.

2.7 *In vitro* infection of cell suspensions

Epidermal cells or MΦ (3×10^6) were incubated with *L. major* amastigotes for 24 h at a ratio of two parasites per cell in 2 ml. For depletion of extracellular parasites, the cultures of parasites and adherent MΦ were washed prior to collection of the cells, and the suspensions of epidermal cells and parasites were incubated with WIC79.3 mAb for 45 min on ice followed by removal of mAb-coated parasites with anti-mouse IgG Ab coupled to magnetic beads (Dynal) using a magnet.

2.8 Double staining of cell suspensions with fluorescent cell linker and acridine orange/ethidium bromide

Epidermal cells or MΦ that had been infected with *L. major in vitro* were labeled with the fluorescent lipo-

philic dye PKH-26 (Zynaxis Cell Science, Malvern, PA) which is stably incorporated and retained in the plasma membrane. Labeling with the fluorescent cell linker kit was performed according to the manufacturer's instructions. Subsequently, 2×10^6 cells were injected i.d. into the footpads of uninfected mice. After 24, 48 or 96 h, the popliteal LN were collected and single cell suspensions were analyzed by fluorescence microscopy for the presence of PKH-labeled cells containing *L. major*. Intracellular parasites were visualized by incubating the LN cells with a mixture of acridine orange (5 μg/ml) and ethidium bromide (50 μg/ml) for 10 min at RT [23].

2.9 Double labeling of LN sections by a combination of immunogold-silver staining and immunoenzymatic labeling

For immunohistological identification of *L. major*-containing cells, tissue samples were snap-frozen in OCT compound (Miles, Naperville, IL). Cryostat sections (5 μm) were thawed onto HCl-rinsed slides, air-dried and fixed in acetone (−20 °C, 10 min). The slides were cleared from OCT compound by washing with a solution containing 5 % skim milk powder and 0.1 % Tween 20 in PBS, pH 7.4, (Blotto) and nonspecific binding sites were blocked by incubation in Blotto supplemented with 20 % FCS (30 min, RT). Mixed labeling of tissue sections was performed as previously described in detail [24]. The following sequence of treatments was used: rabbit anti-*L. major* antiserum (for immunogold-silver staining) and mAb NLDC-145 or mAb anti-Mac-1 (for immunoenzymatic labeling) (overnight, 4 °C); gold-conjugated anti-rabbit Ig Ab (1 h, RT); blocking with Blotto containing 20 % normal rabbit serum (20 min, RT); biotinylated anti-rat Ig Ab (45 min, RT); preformed streptavidin-biotin alkaline phosphatase complex (Dako; 45 min, RT); Fast Blue solution containing 0.2 mg/ml naphtol AS-MX phosphate (Sigma, Deisenhofen, FRG), 1 mM levamisole and 0.2 mg/ml Fast Blue BB salt (Serva, Heidelberg, FRG) in Tris buffer (0.1 M, pH 8.2) (5–10 min); immunogold-silver enhancement mixture (Amersham, Braunschweig, FRG) (5–10 min). The tissue sections were counterstained with nuclear fast red and mounted (Aquatex, Merck, FRG). For evaluation, a microscope equipped for polarized incident light (epipolarization) and interference-contrast microscopy was used (Zeiss Axiophot, Oberkochen, FRG).

2.10 Assay for DTH reactivity

Four days after i.d. inoculation of *L. major* lysate (equivalent to 2×10^6 organisms) or myoglobin (10 μg; Sigma) into the dermis of athymic nude mice, 10^6 cells collected from the regional LN were transferred to euthymic recipient mice. The recipients were challenged 7 days later. A 10-μl volume of *L. major* lysate (equivalent to 10^6 parasites) or myoglobin (5 μg) was injected i.d. into both ears of each mouse, using a 100-μl Hamilton syringe mounted with a 30-gauge Yale needle. The thickness of the ears was measured with an engineering micrometer before and 24 h as well as 48 h after Ag challenge. The degree of swelling was expressed as the difference in thickness of the Ag-injected and the untreated ears. Experimental groups consisted of four to seven mice each.

3 Results

3.1 *L. major*-bearing dendritic cells are detectable in the LN draining the site of infection

Mice were infected i.d. with *L. major* promastigotes and at various time points thereafter, the draining inguinal LN were collected for immunohistological analysis. Parasite-containing cells were identified by a double labeling technique [2, 24] that allows the sensitive and highly specific detection of two types of Ag expressed by the very same cell. As early as 2 to 4 days after infection, small numbers of cells containing *L. major* Ag could be demonstrated in sections of the local LN. Strikingly, parasite staining was associated with distinct clusters of NLDC-145$^+$ dendritic cells (Fig. 1A and B). Double labeling also revealed that the *L. major*-bearing cells were located in the T cell-dependent areas of the LN (Fig. 1C). It is of particular importance that parasite Ag expression at this early stage of infection was exclusively associated with NLDC-145$^+$ dendritic cells, but not with Mac-1$^+$ MΦ. In a time course analysis, *L. major*-containing MΦ could only be detected in a much later phase of infection (Table 1). Prior to appearance of parasitized MΦ, there was a significant increase in the number of uninfected MΦ in the draining LN from 2.6 ± 0.4 on day 4 to 8.5 ± 2.2 on day 14 (cells per random field at × 400 magnification ± SD), indicating that those MΦ have immigrated from the blood rather than the lesion and take up *L. major* in the LN.

To determine whether LN dendritic cells can harbor viable parasites, NLDC-145$^+$ cells were collected 2 days after i.d. infection with *L. major* by treatment with Ab and magnetic beads and were seeded into blood agar cultures. Full growth of parasites occurred and, upon injection into BALB/c mice, those organisms caused the development of progressive lesions (data not shown).

3.2 Epidermal LC infected with *L. major* migrate from the skin to the draining LN

To determine whether the parasite-containing NLDC-145$^+$ dendritic cells in the LN originate from LC in the cutaneous lesion, we assessed the migratory capacity of epidermal LC infected with *L. major*. For this purpose, isolated epider-

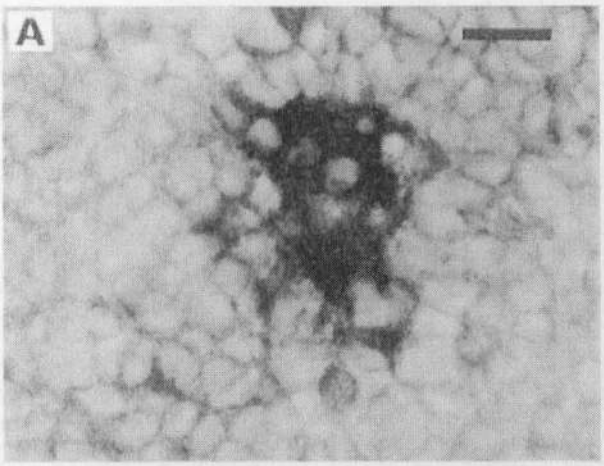
Table 1. *L. major* Ag expression by MΦ and dendritic cells in the LN draining the site of cutaneous infection[a)]

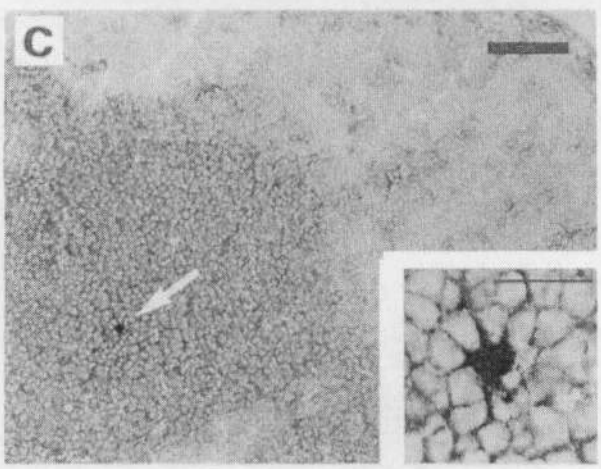
mAb	Expression of *L. major* Ag[b)] on day		
	4	14	24
anti-Mac-1	−	−	+++
NLDC-145	+	++	+++

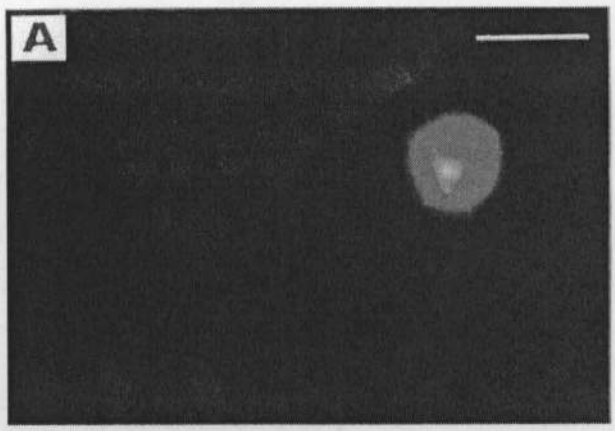
a) At various time points after i.d. infection with 1×10^5 *L. major* promastigotes, serial sections of the draining LN were subjected to double staining for detection of NLDC-145$^+$ or MAC-1$^+$ cells expressing *L. major* Ag (immunoenzymatic and immunogold-silver labeling, see Fig. 1).

b) −: No double-positive cells; +: 1 to 4 clusters; ++: 5 to 10 clusters; +++: more than 10 clusters of double-positive cells per section.

mal cells were incubated with amastigotes *in vitro*. We have previously shown that only LC, but no other cells of the epidermis, are able to take up *L. major* [2]. After removal of extracellular parasites, the epidermal cell suspension was labeled with the fluorescent cell linker PKH-26 and was inoculated into the dermis of uninfected mice. The draining LN cells were collected 24 h, 48 h or 96 h later and the presence of intracellular parasites was assessed by staining with acridine orange and ethidium bromide. Indeed, PKH-26$^+$ epidermal cells could be recovered from the LN,







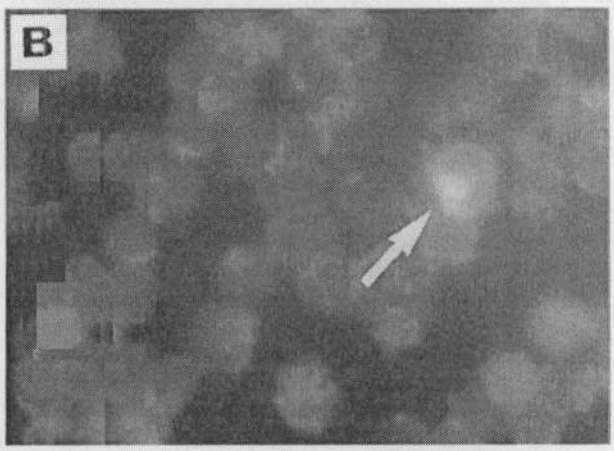


Figure 2. Epidermal LC transport *L. major* from the skin to the regional LN. After *in vitro* infection with *L. major* amastigotes and labeling with the red fluorescent cell linker PKH-26, 2×10^6 epidermal cells were inoculated into the dermis of uninfected mice. The draining LN were collected 24 h later. Single-cell suspensions were stained with acridine orange and ethidium bromide to assess the presence of intracellular parasites and were analyzed by fluorescence microscopy. (A) Micrograph showing a PKH-26-labeled epidermal cell that has migrated into the LN. (B) The same area as in A, demonstrating that the PKH-26$^+$ cell contains an amastigote (arrow). The intracellular organism appears as a yellow spot in A, due to mixing of the red and green fluorescence. Bar = 10 μm.

Figure 1. Dendritic cells expressing *L. major* Ag can be detected in the T cell areas of LN draining the cutaneous lesion. Four days after i.d. infection of mice with 1×10^6 *L. major* promastigotes, the regional LN were collected and analyzed for the presence of parasite-bearing cells, using double labeling immunohistochemistry. (A) The immunoenzymatic labeling shows a characteristic cluster of NLDC-145$^+$ dendritic cells (brightfield illumination). (B) In the same area, *L. major* Ag can be visualized by the accumulations of shining silver grains after labeling with anti-*L-major* antiserum (immunogold-silver staining, epipolarization illumination). (C) Double labeling of LN section with anti-CD4 and anti-CD8 mAb (immunoenzymatic staining) as well as anti-*L. major* antiserum (immunogold-silver staining) reveals that parasite-expressing cells (arrow and, at higher magnification, in inset) are localized in the T-cell areas (brightfield illumination). Bars = 10 μm (A, B, and inset of C), 50 μm (C).

and the majority of these migratory cells (64% ± 9%) were *L. major*-containing LC (Fig. 2). Most importantly, no other cells of the LN (*i.e.* PKH-26$^-$ cells) were ever seen parasitized with *L. major* at these time points. This documents that early in infection only immigrant LC are the source of parasite-bearing cells in the regional LN. The migratory activity of PKH-26$^+$ epidermal cells was abrogated by fixation with 2% glutaraldehyde prior to inoculation into the dermis. On the other hand, migration of non-infected PKH$^+$ epidermal cells was comparable to that of parasite-treated cells, confirming the suggestion of Larsen et al. [9] that the translocation of LC is due to a nonspecific inflammatory response rather than part of the adaptive immune response.

In control experiments, PKH-labeled MΦ that had been infected with *L. major in vitro* and were used according to the same protocol could never be detected in the LN. These findings indicated that epidermal LC, but not MΦ, have the capacity to transport *L. major* from the skin to the draining LN.

3.3 Dendritic cells in the regional LN of i.d. sensitized mice stimulate *L. major*-specific T cells

To assess the capacity of *L. major*-containing migratory dendritic cells to induce a T cell immune response, mice were sensitized i.d. with *L. major* lysate and 4 days thereafter, the draining LN were collected as a source of APC for an *in vitro* T cell assay. It has been shown by immunohistology (Table 1) that at this time point only LN dendritic cells, but not MΦ, express *L. major* Ag. T cells from *L. major*-primed mice were used as effector cells and the cultures were set up in the absence of exogenous Ag. The data in Fig. 3A document that LN cells from *L. major*-treated mice, but not from PBS-treated controls, were indeed able to induce a pronounced proliferation of Ag-primed T cells. Similar results, with a more vigorous proliferative response, were obtained using viable parasites for cutaneous sensitization and cloned *L. major*-reactive T cells as responder cells (Fig. 3B).

To exclude the possibility that APC other than dendritic cells, expressing low amounts of *L. major* Ag and therefore being not detectable by immunocytochemical techniques, were responsible for the stimulation of T cells, NLDC-145$^+$ dendritic cells were depleted from the LN population. Since NLDC-145$^-$ LN cells were not capable of inducing a T cell response (Fig. 3B), we conclude that dendritic cells account for the T cell-stimulatory effect.

3.4 Dendritic cells in the regional LN of i.d. sensitized mice can prime *L. major*-specific T cells *in situ*

Having demonstrated that migratory dendritic cells stimulate *L. major*-specific T cells *in vitro*, we next examined whether they are able to induce primary T cell activation *in situ*. For this purpose, APC in the draining LN of i.d. sensitized mice were tested for their capacity to induce DTH reactivity to *L. major* Ag upon transfer into naive recipient mice. To exclude the transfer of effector T cells, athymic nu/nu mice were used as donors. The results in Fig. 4 show that APC in the draining LN of *L. major*-

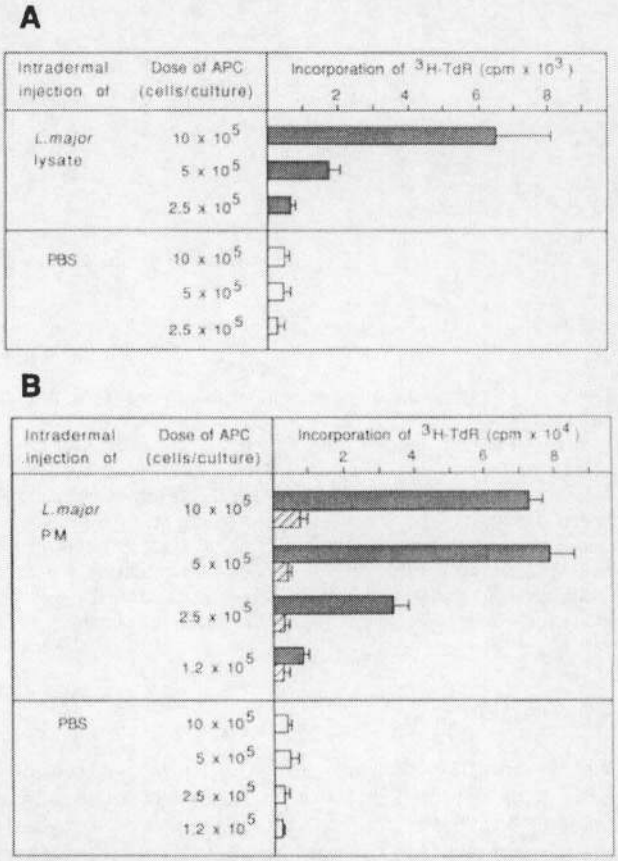


Figure 3. Dendritic cells in the LN of cutaneously sensitized mice stimulate a secondary response of *L. major*-specific T cells. Four days after i.d. injection of *L. major* lysate (A) or virulent promastigotes (PM, B), the draining LN were collected and, after irradiation with 900 rad, were used as a source of APC. The LN cells were either unselected (dark bars) or depleted of NLDC-145$^+$ dendritic cells (hatched bars). For the controls, LN cells were obtained from mice that had received an i.d. injection of PBS (white bars). Various numbers of LN cells were cultured with T cells from *L. major*-primed mice at a dose of 1 × 10^5/microwell (A) or with *L. major*-specific cloned T cells at a dose of 2 × 10^4/microwell (B). Incorporation of [^{3}H]dThd was determined at 60 to 78 h (A) or at 48 to 66 h (B). The data represent means ± SD of triplicate cultures. The background proliferation of T cells was below 500 cpm and below 2000 cpm for (A) and (B), respectively.

sensitized nude mice were able to induce a significant DTH response, as determined by the ear swelling of the recipients after local challenge with *L. major* Ag (compare group 1 with group 2). The great efficiency of migratory dendritic cells can be estimated by comparing the magnitude of their effect with the swelling induced in mice that had been sensitized not by cell transfer but by infection with *L. major* for as long as 30 days (group 6). Again, depletion of dendritic cells from the transferred LN population completely abrogated the stimulatory effect.

In addition, we verified the Ag specificity of the DTH response. It was demonstrated that APC in the local LN of *L. major*-sensitized mice, although capable of inducing DTH reactivity to parasite lysate, did not stimulate a DTH response to the irrelevant Ag myoglobin (compare group 1 with group 3). Thus, the *L. major*-bearing dendritic cells do not induce an unspecific stimulation of T cells *in situ*.

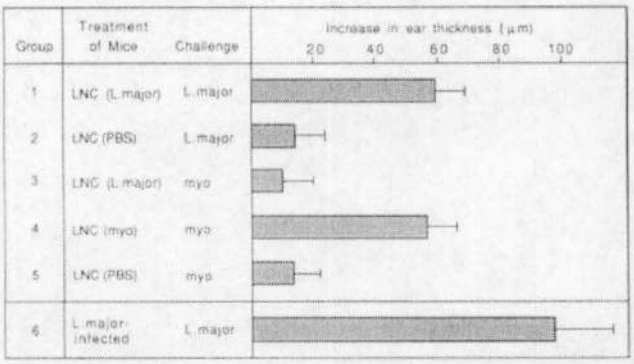


Figure 4. Migratory dendritic cells can prime mice for elicitation of an *L. major*-specific DTH reaction. Four days after inoculation of *L. major* lysate (groups 1 and 3), PBS (groups 2 and 5), or myoglobin (myo, group 4) into the dermis of athymic nu/nu mice, regional LN cells (LNC) were transferred to euthymic recipients. Seven days later, the recipients were challenged with *L. major* lysate or myoglobin in the ear and the local swelling was determined after another 48 h. *L. major*-infected mice challenged with parasite Ag were used as a positive control (group 6). *Horizontal lines* indicate the SD of the arithmetic mean.

4 Discussion

We have recently demonstrated that *L. major*-containing LC can be detected in the cutaneous lesions of infected mice [2]. Strikingly, we observed a pronounced decrease of the number of LC in the epidermis overlying the parasite-containing dermal infiltrate and a concomittant increase of LC in this area of the dermis, some of which contained *L. major*. No parasitized LC were ever observed in the epidermis. These findings suggested that LC migrate from the epidermis into the dermis prior to the uptake of *L. major*. It was therefore of interest to analyze whether those LC have the capacity of further movement to the draining LN.

Previous evidence for migration of LC from the epidermis to regional LN has been derived from contact sensitization and skin transplantation experiments [8, 10, 25]. However, it is difficult to exclude that contact allergens, due to their small size, get to the LN independently and are taken up by resident dendritic cells, or that the effect is caused by dermal dendritic cells. In the current study, the use of LC infected with the obligatory intracellular parasite *L. major* and labeled with a stable fluorescent cell linker allowed us to demonstrate directly the migration of epidermal LC into the LN. This finding strongly suggests that *L. major*-bearing dendritic cells in the LN draining the site of cutaneous infection are derived from LC that originate in the epidermis and, as indicated by our previous work summarized above [2], have taken up parasites en route in the dermal compartment.

Dendritic cells in the regional LN of *L. major*-infected mice were potent stimulators of Ag-primed T cells. This is reminiscent of our earlier findings with epidermal LC exposed to *L. major in vitro* [3], and hence corroborates the suggestion that *L. major*-bearing dendritic APC in the draining LN are derived from epidermal LC, a differentiation process that can be reproduced by *in vitro* culture of LC [26]. In addition, the present data show that dendritic cells in the draining LN of i.d. sensitized mice have the ability to prime mice *in situ* for elicitation of an *L. major*-specific DTH response. Thus, the functions of dendritic cells in cutaneous leishmaniasis are clearly distinct from those of MΦ. MΦ were not seen to transport *L. major* Ag from skin to LN and can not induce a primary T cell immune response [27].

The number of *L. major*-bearing dendritic cells that was responsible for the observed T cell stimulation was very low. Maximal responses could be induced with 5×10^5 to 10×10^5 draining LN cells containing less than 1% dendritic cells, a minor proportion of which expressed *L. major* Ag. Similarly, a small number of *L. major*-laden LC appears to leave the site of cutaneous infection for homing to the LN, because only 0.1 to 0.5% of *L. major*-infected LC labeled with fluorescent cell linker could be recovered from the draining LN. This is in line with observations reported for other experimental systems [27, 28]. The great efficiency of the migratory dendritic APC is presumably based on their capacity of binding and activating large numbers of Ag-specific T cells in the draining LN [5, 6]. Another crucial feature related to the migratory and stimulatory function of LC is their unique ability to retain Ag in an immunogenic form for at least 2 days. This is effected by down-regulating the synthesis of MHC class II molecules during LC differentiation, and, consequently, a prolonged half-life of MHC class II/peptide complexes, as compared with MΦ [29–31].

The signals promoting the migration of LC have not been defined although the involvement of TNF-α [32] and IL-1β [33, 34] has been proposed. It is likely that several cytokines as well as adhesion molecules act in concert. As shown in the current study and by others [9, 35], the presence of Ag in the LC is not essential for movement of the cells. Thus, the migration and differentiation of LC seems to be part of a nonspecific local inflammatory response. An important question arising is that of the ultimate fate of *L. major*-bearing LC after they gained entry into a regional LN and have stimulated resting T cells. It is quite unlikely that they recirculate because dendritic cells cannot be found in the efferent lymph [5]. Because infected LC were found to restrain intracellular parasite replication *in vitro* by an as yet unknown mechanism [2] it is possible that *L. major*-laden immigrant LC may dwell in the draining LN for some time.

It is noteworthy that transport of *L. major* by LC could be documented both in susceptible BALB/c mice and in resistant C57BL/6 mice (results not shown) and thus appears to be a general mechanism for initiation of the parasite-specific immune response. It will be of interest to analyze whether presentation of *L. major* Ag by dendritic cells favors the development of particular T cell subsets. If this is the case, the initial predominance of *L. major*-bearing dendritic cells in the draining LN of both strains of mice may explain our previous observation that, at the early stage of infection, the frequency of IL-4-secreting LN cells was similar in BALB/c and C57BL/6 mice [36]. In addition, the availability of co-stimulatory signals in the microenvironment (*e.g.*, cytokines) may influence the nature of the T cell immune response [37].

The results of this study complete our preceding work [2, 3] and are consistent with the hypothesis that on inoculation

of *L. major* into the skin, LC migrate from the epidermis to the infected dermis and take up parasites. In contrast to MΦ which serve as scavengers and are the predominant site of parasite replication, the primary function of *L. major*-infected LC appears to be the transport of parasite Ag to the draining LN. During migration, those LC differentiate into potent APC with the ability to initiate the *L. major*-specific cellular immune response by stimulation of resting T cells. Furthermore, we propose that *L. major*-infected LC that remain in the dermis, in addition to MΦ, present parasite Ag to T cells infiltrating the lesion. The relative frequency of the two types of APC as well as the pattern of locally available cytokines may influence the kind of T cell immune response that will predominate.

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