Protozoan parasites of the genus *Leishmania* are transmitted to mammalian hosts by phlebotomine sand flies. During the blood meal of an infected insect, the flagellated promastigote form gains access to host tissues and is rapidly phagocytosed, predominantly by macrophages in which the parasites transform into amastigotes devoid of the external flagellum. This process occurs within organelles termed parasitophorous vacuoles (PV). Amastigotes are well adapted to the conditions in the PV, allowing them to survive and multiply intracellularly (7).

A critical part of the chain of events leading to degradation and death of intracellular microorganisms, including *Leishmania* sp. parasites, in macrophages is phagolysosome biogenesis. Newly formed phagosomes are unable to digest their contents and must therefore undergo a complex maturation process, which is driven by a series of sequential fusion events between phagosomes and early endosomes, late endosomes, and lysosomes (1, 10, 14). Each phagosome maturation stage is characterized by specific compartmental markers, such as the endosomal markers CD71 and Rab4, the late endosomal markers Rab7 and CD68, and the late endosomal/lysosomal glycoproteins LAMP-1 and LAMP-2. These steps lead to the formation of the phagolysosome, an acidic compartment displaying the harsh and lytic environment needed to destroy microorganisms and process microbial antigens for presentation to the specific immune system.

To date, the information on PV harboring *Leishmania* sp. is derived almost exclusively from studies with macrophages. However, we (3, 25, 32) and others (15, 18, 21, 27, 31) have shown that dendritic cells (DC) also internalize *Leishmania* sp. parasites and are the most potent antigen-presenting cells for initiation and regulation of the specific T-cell response. The properties of DC vary with the different stages of their life span. DC in nonlymphoid tissues are immature cells which can phagocytose and process particles but are only weak stimulators of T-cell immune responses. The differentiation of DC is triggered by exposure to pathogens or inflammatory cytokines. It is accompanied by a loss of endocytic activity and a marked upregulation of their antigen presentation and costimulatory functions (2, 13). Although it has been demonstrated that *Leishmania* sp. parasites internalized by DC reside within endocytic vacuoles (11, 15, 27), the endosomal trafficking routes in DC and the remodeling of the endosome/lysosome compartment of infected DC during their maturation are largely unknown.

In the present study, we used *L. major*, a cause of human cutaneous leishmaniasis, to analyze the properties of parasite-containing PV in DC of different maturation stages. Phagolysosome biogenesis was assessed by fluorescence labeling of endocytic marker proteins and confocal laser microscopy. Furthermore, we characterized the fusion competence of PV harboring *L. major* in DC, and we examined whether different life cycle stages of *L. major* induce distinct pathways of phagosome maturation in DC. The results show that *L. major*-harboring PV in DC did not acquire the whole array of molecules typical
for lysosomal organelles and had a reduced capacity to fuse with lysosomes. The impaired fusion activity was most pronounced in immature DC. Moreover, our data demonstrate that the highest level of inhibition of endosomal-lysosome fusion in immature DC was induced by live promastigotes, whereas killed organisms were not effective and amastigotes established only a partial block of PV fusion with lysosomes.

**MATERIALS AND METHODS**

**Generation and culture of DC.** Freshly prepared DC were generated from the bone marrow of 6- to 8-week-old BALB/c mice (Charles River Breeding Laboratories, Sulzfeld, Germany) as described previously (20, 28). Briefly, 2 × 10^6 bone marrow cells derived from the tibia and femur of the hind legs were incubated in 10 ml RPMI 1640 medium (Invitrogen, Karlsruhe, Germany) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum, 2 mM l-glutamine, 10 mM HEPES buffer, 0.05 mM β-mercaptoethanol, 100 μM penicillin, and 20 μg/ml gentamicin (culture medium) in the presence of 200 U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF; PeproTech, London, United Kingdom). Cultures were fed with GM-CSF on day 3 if maintained for a period of 3 to 8 days and were fed again on day 6 or on days 6 and 8 if maintained for a period of 8 or 10 days, respectively. The bone marrow-derived DC (BMDC) were harvested on day 6, 8, or 10 and were allowed to adhere to glass chamber slides (Nunc, Wiesbaden, Germany) for 3 to 4 h. The cells were shown to have a typical DC morphology with a myeloid DC phenotype (mch class II, CD80, CD86, CD11c, CD205, B220, and CD11b). For activation, BMDC were harvested on day 6 or on days 6 and 8 with l-glutamine-coupled dextran (LPS; Sigma-Aldrich, Taufkirchen, Germany) at 1 μg/ml, Cpg oligodeoxynucleotide (ODN) 1668 (5'-TCC ATG ACG TTC CTG ATG CT-3') or the control AT-rich ODN (non-Cpg ODN, 5'-ATT ATT ATT ATT ATT 3') (QIAGEN Operations, Cologne, Germany) at 100 μg/ml, CD40 ligand (PeproTech) at 1 μg/ml, or interleukin 10 (IL-10; PeproTech) at 20 μg/ml. A mouse DC line derived from fetal skin (FSDC) (12) was passaged in culture medium. All experiments were done with subconfluent cultures.

**Parasites and treatment of DC with L. major.** The cloned virulent L. major isolate (HM01/L.81/FE/BNI) was maintained by passage in BALB/c mice. Amastigotes were isolated from skin lesions as described previously (4). Promastigotes were grown in vitro in blood agar cultures. For analysis by confocal microscopy, parasites were stained with green fluorescent cell tracker 5-chloro-10,10,10-trifluoromethoxybenzoylaminotetramethylrhodamine (CMTMR) (Molecular Probes) and rhodamine-conjugated rat anti-mouse LAMP-1 MAb (Serotec, Oxford, United Kingdom). Isotype-matched control antibodies were purchased from BD Pharmingen and DAKO/Cytomation (Glostrup, Denmark). After treatment with the primary antibodies, the cells were washed three times with PBS containing Ca^{2+} and Mg^{2+} and were stained with the secondary reagents streptavidin-Alexa Fluor 546 (Molecular Probes) or rhodamine-conjugated rat anti-rabbit antibody (Dianova, Hamburg, Germany) in saponin-containing blocking solution for 45 min at room temperature. Cells were mounted in fluorescent mounting medium (DakoCytomation) before observation with a LSM 510 Zeiss confocal laser scanning microscope. Confocal microscopy images were acquired by use of LSM 510 software and analyzed by use of LSM 3.2 image browser before being exported to Adobe Photoshop. For each experiment and at each time point, the percentages of parasite-containing compartments displaying endocytic marker molecules or having fused with lysosomes were determined after 50 to 300 PV were counted.

**Extracellular staining of DC.** In freshly prepared BMDC populations, DC were identified by labeling with MAb H5/1.1, a hamster IgG directed against mouse CD11c (BD Pharmingen) and cytochrome c-conjugated goat anti-hamster IgG (Dianova) before permeabilization for intracellular staining.

**Statistical analysis.** Unpaired Student's t test was used for statistical analyses. Differences were considered significant when P < 0.05.

**RESULTS**

*L. major* promastigotes reside in late endosomal compartments of DC precursors and immature DC. The phagocytic activity of DC varies with their maturation stage. While mature DC have a strongly reduced ability to internalize particles, DC precursors and immature DC efficiently take up microorganisms, such as *L. major*. For the characterization of *L. major*-containing compartments in DC, we therefore used the cell line FSDC (representing myeloid DC progenitors arrested at an early stage of development) and freshly prepared immature DC (BMDC that had been cultured in the presence of GMP-CSF for 8 days). The features of PV harboring *L. major* in DC were evaluated according to the presence of endosomal marker proteins using intracellular immunostaining and confocal immunofluorescence microscopy. Freshly prepared DC were identified by cell surface labeling of CD11c, and parasites were visualized after pretreating with CMFDA or CMTMR.

After infection of DC with *L. major* promastigotes, all parasites were located in membrane-bound compartments. The outline of the phagosome membrane was largely congruent with the shape of the parasites. Late endosomal/lysosomal molecules could be detected in the majority of PV membranes of both DC precursors and immature DC (Fig. 1). Distinct red rims indicated the presence of CD68 (Fig. 1A, 1 to 5, and Fig. 1B, 1 to 4) and LAMP-1 (Fig. 1A, 6 to 9, and Fig. 1B, 5 to 8) in the membranes of *L. major*-containing organelles in FSDC and BMDC. For examination of the PV biogenesis, BMDC and FSDC were evaluated at 0.5, 1, 4, or 24 h after infection of fluorescent tracer molecules in lysosomes. Subsequently, the cells were exposed to CMFDA-stained parasites at 37°C for 30 min. Thereafter, they were washed with PBS to remove free parasites and were cultured for a further 23.5 h.

**Intracellular immunostaining of proteins of the PV and analysis by confocal microscopy.** At different time points (0.5, 1, 2, 4, or 24 h) after DC exposure to stationary-phase promastigotes for 30 min (see above), the cells were washed with PBS containing 0.25 mM MgCl_2 and 0.35 mM CaCl_2, fixed with 4% paraformaldehyde, and then permeabilized with 0.2% saponin in blocking solution (10% goat serum in PBS containing Mg^{2+} and Ca^{2+}). Thereafter, DC were incubated for 45 min at room temperature in a humidified chamber with one of the following primary antibodies in saponin-containing blocking solution: monoclonal antibody (MAb) C2, a rat immunoglobulin G1 (IgG1) recognizing mouse CD71 (transferrin receptor) (BD Pharmingen, Heidelberg, Germany), fluorescein isothiocyanate-conjugated rat anti-mouse LAMP-2 MAb (BD Pharmingen), rabbit anti-mouse Rab4 antibodies (Santa Cruz, Santa Cruz, Calif.), rabbit anti-mouse Rab7 antibodies (Santa Cruz), phycocerythrin-conjugated rat anti-mouse LAMP-1 MAb (Santa Cruz), and biotin-conjugated rat anti-mouse CD68 (mouse) (MAb) C2, a rat immunoglobulin G1 (IgG1) recognizing mouse CD71 (transferrin receptor) (BD Pharmingen, Heidelberg, Germany), fluorescein isothiocyanate-conjugated rat anti-mouse LAMP-2 MAb (BD Pharmingen), rabbit anti-mouse Rab4 antibodies (Santa Cruz, Santa Cruz, Calif.), rabbit anti-mouse Rab7 antibodies (Santa Cruz), phycocerythrin-conjugated rat anti-mouse LAMP-1 MAb (Santa Cruz), and biotin-conjugated rat anti-mouse CD68 (mac-rosialin) MAb (Serotec, Oxford, United Kingdom). Isotype-matched control antibodies were purchased from BD Pharmingen and DAKO/Cytomation (Glostrup, Denmark). After treatment with the primary antibodies, the cells were washed three times with PBS containing Ca^{2+} and Mg^{2+} and were stained with the secondary reagents streptavidin-Alexa Fluor 546 (Molecular Probes) or rhodamine-conjugated rat anti-rabbit antibody (Dianova, Hamburg, Germany) in saponin-containing blocking solution for 45 min at room temperature. Cells were mounted in fluorescent mounting medium (DAKO/Cytomation) before observation with a LSM 510 Zeiss confocal laser scanning microscope. Confocal microscopy images were acquired by use of LSM 510 software and analyzed by use of LSM 3.2 image browser before being exported to Adobe Photoshop. For each experiment and at each time point, the percentages of parasite-containing compartments displaying endocytic marker molecules or having fused with lysosomes were determined after 50 to 300 PV were counted.
with *L. major*. The results of the immunofluorescence analysis showed that the early endosomal marker CD71 was detectable in few PV (about 10%) only within 30 min (BMDC) or 4 h (FSDC) after infection (Fig. 2). At 24 h, virtually none of the PV in BMDC (<1%) and less than 3% of the PV in FSDC contained this early marker. In contrast, the majority of PV in both FSDC and BMDC expressed LAMP-1 and LAMP-2 proteins already in the early phase of infection. Thus, PV maturation in *L. major*-infected DC seems to occur very fast. The rapid formation of late endosomal compartments was most pronounced in BMDC, as indicated by the early expression of high levels of LAMP-1, LAMP-2, and CD68 (Fig. 2A), while lower amounts of these markers were detected in FSDC (Fig. 2B). On the other hand, a higher proportion of PV in FSDC was positive for Rab7, another late endosomal molecule, which is involved in the fusion of late endosomes with lysosomes (5, 22). Of note, the immortalized line FSDC has been shown to display the morphology and surface marker profile suggestive of a myeloid progenitor cell with features of both DC and macrophages (12).

**Influence of the maturation and activation status of DC on PV biogenesis.** The results described above demonstrated that
L. major-containing PV in a DC precursor line resemble those in immature DC with regard to their ability to fuse rapidly with late endocytic compartments. Next, we compared the characteristics of PV in freshly prepared immature and mature BMDC at different times after infection with L. major.

High levels of the late endosomal markers CD68, LAMP-1, and LAMP-2 were present in the parasite-containing organelles of both immature and mature DC (Fig. 3), even at the earliest time points after infection, indicating that the rapid formation of late endosomal compartments harboring L. major in DC is independent of the stage of DC maturation. The most notable difference between mature and immature BMDC was the rapid acquisition of Rab7 by L. major-containing PV in mature DC (Fig. 3B). While Rab7, a major effector of late endosome/lysosome transformation, could not be detected in PV of immature DC at any of the studied time points after infection (30 min to 4 h), 22% of the parasite-harboring compartments in mature DC displayed this molecule already at 30 min postinfection. At 1 h, the percentage of Rab7-positive PV had decreased considerably, and only background levels were detectable thereafter (Fig. 3B). The absence of Rab7 in immature BMDC suggests an arrest of their PV biogenesis at the stage of late endosomes.

The properties of DC not only change during their maturation but also vary depending on their activation status. To determine the effect of DC activation on the biogenesis of L. major promastigote-containing PV, BMDC were exposed to the stimulus LPS, CpG ODN, or CD40 ligand and were compared with nonstimulated BMDC with regard to the presence of endosomal/lysosomal marker proteins in the PV at 24 h after infection. Furthermore, the effect of IL-10, a cytokine promoting the development of tolerance-inducing DC, was examined. The results demonstrated that none of these DC treatments modified the biogenesis of L. major-harboring PV in BMDC (data not shown). Together, our findings document that PV biogenesis is influenced by the stage of maturation but not the activation status of DC.

The fusion activity of L. major-containing PV with lysosomes is strongly reduced in immature DC. The finding that Rab7, a key molecule controlling the formation of phagolysosomes, is not present in PV of immature BMDC and can be detected only during the initial phase of infection in PV of mature BMDC (see above) (Fig. 3) suggested that L. major modulates the fusogenic activity of phagosomes in DC. To test this assumption, BMDC at different stages of maturation were in-
fected with CMFDA-labeled *L. major* promastigotes, and the lysosomes were stained with LysoTracker dye. Subsequently, the fusion activity of the PV with lysosomes was analyzed by confocal microscopy at 24 h after infection. *L. major*-harboring PV from immature BMDC rarely fused with lysosomes (Fig. 4, panels A and B1); only about 5% of the lysosomal compart-
ments in BMDC that had been cultured for 6 days colocalized with promastigotes. During further DC maturation, the percentage of fusion events increased and reached a level of about 18% (Fig. 4, panels A and B2 to B5). The poor fusion activity of promastigote-containing organelles with lysosomes in immature DC cannot be explained by a delay in PV maturation, because fusion events were almost absent even after 2 and 3 days of infection (data not shown).

Interestingly, the number of parasites per infected DC at 72 h after pulse infection with promastigotes was slightly higher in immature BMDC than in mature BMDC (immature BMDC, 2.56 parasites per DC; mature BMDC, 2.23 parasites per DC), while it was almost identical at 24 h (immature

**FIG. 4.** Fusion properties of *L. major*-containing PV in BMDC at different maturation stages. BMDC that had been cultured for 6, 8, or 10 days were exposed to CMFDA-labeled *L. major* promastigotes for 30 min. Subsequently, LysoTracker dye was added to stain the lysosomes. After 24 h, the cells were fixed and analyzed for lysosomes colocalizing with parasites by confocal fluorescence microscopy. DC were identified by CD11c labeling (blue). (A) For each BMDC population, the percentage of parasite-containing lysosomal compartments (fusion events) was determined after 50 to 200 organelles were counted. Data are the mean values ± standard deviations for two independent experiments. Panel B1, *L. major*-containing PV (green staining) in immature BMDC (cultured for 6 days) not colocalizing with lysosomes (red staining). Panels B2 to B5, *L. major*-containing PV (green staining) in mature BMDC (cultured for 10 days) that colocalizes with a lysosomal compartment (arrows). Panel B3 is a differential interference contrast image; panel B5 represents the superimposition of the fluorescence images shown in panels B2 and B4. Yellow color indicates colocalization. Optical sections (1-μm thickness) are shown.

Only live promastigotes, not heat-killed promastigotes or amastigotes, cause a block of fusion between PV and lysosomes in DC. To evaluate whether the incompetence of *L. major*-harboring PV to fuse with lysosomes depends on parasite viability or life cycle stage, immature BMDC or FSDC were exposed to live promastigotes, heat-killed promastigotes, or live amastigotes. In contrast to PV containing live promastigotes, a large proportion of PV containing heat-killed promastigotes (about 70%) colocalized with lysosomes in both BMDC and FSDC (Fig. 5). Moreover, the fusogenic activity of PV containing amastigotes was much higher than that of PV containing live promastigotes (34% compared to 10% in freshly prepared DC). The percentage of fusion events was higher (20%) in the DC progenitor line, FSDC, that had been exposed to live promastigotes, a finding that correlated with the higher level of Rab7 in the PV of these cells (see above) (Fig.
2). These observations suggest that after internalization of live promastigotes, the maturation of PV in DC is strongly reduced and the vast majority of PV is arrested at the stage of late endosomes. In contrast, a considerable proportion of PV containing amastigotes fuse with lysosomes.

**DISCUSSION**

DC are extraordinarily efficient in processing microbial antigen and presenting it to T cells, thus linking the innate and adaptive arms of the immune system. In leishmaniasis, the initiation and instruction of the specific immune response by DC is critical for the outcome of the disease. An intriguing feature of DC is the tight control of their antigen processing and presentation functions according to their maturation status. Although it has been shown previously that Leishmania spp. parasites reside in endosomal compartments of DC (11, 15, 27), the characteristics of Leishmania spp.-containing PV in DC remain poorly understood, and it is unknown whether they are subject to changes during DC maturation. In the present study, the major new findings are that the fusogenic activity of L. major-harboring compartments toward lysosomes is strongly impaired in immature DC and that this inhibition of phagosome-lysosome fusion is dependent on the viability and life cycle stage of the parasite.

Both macrophages and DC serve as host cells for Leishmania spp. parasites, but the two cell types differ in important ways. DC have unique surveillance and migratory properties, enabling them to carry parasite antigens captured in the infected skin to the draining lymph nodes for presentation to naive T cells, thus initiating the specific immune response (23, 24). A critical specialization of DC is the process of maturation (2). Immature DC efficiently take up antigen but express relatively low levels of major histocompatibility complex (MHC) and costimulatory molecules on their surface and, thus, have a reduced capacity for T-cell stimulation. In contrast, mature DC downregulate their endocytic activity, while the transport of immunogenic peptide-MHC class II complexes from the lysosomal compartment to the plasma membrane, together with increased surface expression of costimulatory molecules, enables them to activate even resting T cells (6, 16, 30). Using L. major-infected Langerhans cells, DC derived from the skin, we previously demonstrated that the MHC class II levels associated with L. major-containing PV decrease with DC maturation (11). We also showed that immunogenic peptide-MHC class II complexes in L. major-infected DC are remarkably long-lived (11), supporting the concept that DC are highly specialized for the prolonged retention and efficient presentation of parasite antigen in leishmaniasis.

In the present study, we analyzed the biogenesis of PV housing L. major, as characterized by the acquisition of endosomal and lysosomal marker proteins, and the fusion activity of the L. major-containing PV with lysosomes in DC at different maturation stages. The determination of the kinetics of endosome/lysosome marker recruitment into the PV revealed that L. major-containing phagosomes in all types of DC tested, a DC precursor line as well as freshly prepared immature and mature DC, can fuse with late endocytic compartments very quickly after their formation, as hallmarked by the rapid acquisition of high levels of LAMP-1, LAMP-2, and CD68 (Fig. 2 and 3). On the other hand, none or only very small amounts of the PV were found to display the early endosomal molecules CD71 and Rab4, indicating low or very transient interactions with early endocytic organelles just upon parasite uptake. Interestingly, very similar patterns of endosomal/lysosomal marker expression were observed with DC that, in addition to culture in the presence of GM-CSF, were treated with the stimuli LPS, CpG ODN, or CD40 ligand. This finding supports the view that activation of DC, although greatly enhancing their T-cell-stimulatory capacity via induction of IL-12 expression (17, 21), does not influence the biogenesis of L. major-containing phagosomes.

A most important feature was the developmental regulation of Rab7 expression in L. major-containing phagosomes of DC. This protein, which controls the fusion of late endosomes with lysosomes, was virtually absent in PV of immature DC, but significant amounts were detected in those of DC precursors and mature DC. It is noteworthy that the maximal levels of Rab7 expression in DC were consistently lower than those reported for macrophages (7). An analysis of the fusion activity of L. major-containing PV with lysosomes demonstrated that the fusogenic property was strongly impaired in freshly prepared immature DC infected with promastigotes, while it increased steadily during DC maturation (Fig. 4). Interestingly, the decreased fusion activity of PV with lysosomes in immature DC correlated with enhanced parasite survival in these cells. Thus, L. major promastigotes reduce lysosomal degradation in immature DC. In vivo, DC in the periphery, such as skin, generally exhibit an immature phenotype. The observation that PV of immature DC, relative to those of mature DC, fuse poorly with lysosomes provides an explanation for how immature DC, while migrating from the infected skin to the draining lymph node and undergoing the maturation process, can pre-
serve parasite antigen for the generation of peptide-MHC complexes and efficient presentation to T cells.

The results of the present study also show that the inhibition of PV-lysosome fusion in *L. major*-infected DC is dependent upon the viability and life cycle stage of the parasite. Live promastigotes blocked the phagolysosome generation in immature BMDC almost completely (Fig. 4 and 5), whereas promastigotes blocked the phagolysosome generation in immature BMDC almost completely (Fig. 4 and 5), whereas phagosomes containing killed organisms showed extensive fusion with lysosomes. Furthermore, the low level of Rab7 expression in the *L. major*-containing PV of DC was a specific effect of live parasites. While Rab7 could not be detected in PV of immature DC and was present in 22% of the PV in mature DC treated with live promastigotes, a large proportion of PV in immature DC and was present in 22% of the PV in mature DC treated with live promastigotes, a large proportion of PV in immature DC (53%) and mature DC (63%) that had been exposed to heat-killed parasites expressed Rab7 (Fig. 3 and data not shown). *L. major* amastigotes induced a considerably higher level of fusion activity than promastigotes. These findings are reminiscent of the situation with macrophages, for which it has been demonstrated that promastigotes, unlike amastigotes, are located in phagosomes that poorly fuse with late endocytic/lysosomal compartments. This phenomenon could be linked to the stage-specific expression of lipophosphoglycan, the predominant surface glycoconjugate of promastigotes (8, 9, 19). The promastigote-phagocytosing phagosomes with low fusogenic activity were shown to display an impaired recruitment of Rab7 (29). Again, the latter finding with infected macrophages is analogous to our observation with DC and suggests that, in both types of host cells, the fusion restriction allows the differentiation of promastigotes into amastigotes which are more adapted to the lysosomal compartment. However, an important characteristic of the inhibition of phagosome-lysosome fusion in DC is its regulation according to the stage of DC maturation.

In conclusion, the findings of the present study extend our understanding of the interaction of DC with *Leishmania* sp. and the mechanisms underlying the unique ability of DC to initiate the parasite-specific T-cell response so efficiently. Our data are consistent with the concept that immature DC in the skin, upon ingestion of the promastigote form deposited by the sand fly vector, can sequester antigen by reducing lysosomal degradation until the maturing cells have migrated to the draining lymph node. When reaching the lymph node, lysosomal proteolysis in mature DC mobilizes the formation of immunogenic peptides to be loaded onto MHC class II molecules and presented to resting T cells. Immature DC that remain in the skin and mature in response to local inflammatory signals may also present antigenic peptides derived from killed promastigotes or amastigotes that are released by macrophages, thus contributing to the instruction of the local immune response.

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