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Cutting Edge: Neutrophil Granulocyte Serves as a Vector for Leishmania Entry into Macrophages

Ger van Zandbergen, Matthias Klinger, Antje Mueller, Sonja Dannenberg, Andreas Gebert, Werner Solbach, and Tamás Laskay

Macrophages (MF) are the final host cells for multiplication of the intracellular parasite Leishmania major (L. major). However, polymorphonuclear neutrophil granulocytes (PMN), not MF, are the first leukocytes that migrate to the site of infection and encounter the parasites. Our previous studies indicated that PMN phagocyte but do not kill L. major. Upon infection with Leishmania, apoptosis of human PMN is delayed and takes 2 days to occur. Infected PMN were found to secrete high levels of the chemokine MIP-1β, which attracts MF. In this study, we investigated whether MF can ingest parasite-infected PMN. We observed that MF readily phagocytosed infected apoptotic PMN. Leishmania internalized by this indirect way survived and multiplied in MF. Moreover, ingestion of apoptotic infected PMN resulted in release of the anti-inflammatory cytokine TGF-β by MF. These data indicate that Leishmania can misuse granulocytes as a “Trojan horse” to enter their final host cells “silently” and unrecognized. The Journal of Immunology, 2004, 173: 6521–6525.

Leishmaniasis is caused by the cutaneous infection with promastigotes of the genus Leishmania. In the mammalian hosts Leishmania are obligate intracellular pathogens. After infection, most Leishmania promastigotes are rapidly killed in the extracellular tissue environment. However, some can escape the toxic extracellular milieu and survive if they gain access to phagocytic cells (1). The first phagocytic cells that infiltrate the s.c. site of experimental infection with 1–2 × 10⁶ Leishmania promastigotes are neutrophilic granulocytes (polymorphonuclear neutrophil granulocytes (PMN))³, followed by a wave of macrophages (MF) about 2 days later (2). PMN can internalize Leishmania promastigotes (3). Importantly, inside PMN the parasites can survive but no multiplication of the parasites has been observed (3, 4). Therefore, these cells might serve solely as temporary host cells for the parasites within the first hours/days after infection (3, 5).

PMN have a very limited life span and undergo spontaneous apoptosis within 6–12 h. We have shown previously that infection with Leishmania delays the apoptotic death program of PMN up to 42 h and, therefore, promotes longevity (5). However, after 42 h even the infected PMN undergo apoptosis (5). The time point at which infected PMN become apoptotic coincides with the peak migration of MF into the infected tissue (2). Thus, in situ, MF would encounter apoptotic PMN harboring intracellular parasites rather than free extracellular Leishmania promastigotes.

The purpose of this study was to investigate whether MF could phagocytose parasitized PMN and to analyze the fate of intracellular Leishmania in both PMN and MF. We found that inside PMN, Leishmania did not multiply and remained in their promastigote form. This is in strong contrast to the multiplicative amastigote form that develops upon infection of MF (1). The infection of PMN induced the release of monocyte-attracting chemotactic factors such as MIP1-β. Although infection with Leishmania delayed their apoptosis, even infected PMN became apoptotic 42 h after infection. We observed that MF ingests infected apoptotic PMN. This process induced the release of the anti-inflammatory cytokine TGF-β by MF. Most importantly, Leishmania parasites that entered MF via the uptake of infected apoptotic PMN survived and multiplied in MF. These findings indicate a new mechanism for Leishmania entry into MF. The parasites use PMN as intermediate hosts and modulate their spontaneous apoptosis and their capacity to attract MF. The data suggest that Leishmania parasites use PMN as “Trojan horses” to enter MF.

Materials and Methods
Leishmania major promastigotes and infection of human peripheral blood neutrophils

Stationary phase L. major (MHOM/IL/81/FEBNI) promastigotes were collected from in vitro cultures in biphasic NNN blood agar medium. Neutrophil granulocytes were isolated from buffy coat blood obtained from healthy adult volunteers as previously described (6). The purity of granulocytes

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³Abbreviations used in this paper: PMN, polymorphonuclear neutrophil granulocyte; MF, macrophage.
was always >99% as determined microscopically after Giemsa staining of cytocentrifuge slides (Thermo Scientific, Pittsburgh, PA). The viability of cells was >99% as assessed by trypan blue dye exclusion. PMN (1 × 10⁷/ml) were cocultivated with *L. major* promastigotes at 37°C at a parasite to PMN ratio of 5:1 in complete RPMI 1640 medium (Invitrogen Life Technologies, Grand Island, NY), supplemented with 10% heat inactivated FCS, 50 μM 2-mercaptoethanol (2-ME), 2 mM L-glutamine, 10 mM HEPES, 100 μg/ml penicillin, and 160 μg/ml gentamicin, all obtained from Seromed-Biochrom (Berlin, Germany). Extracellular parasites were removed either 3 h after coinoculation (for the assessment of parasite survival) or 42 h after coinoculation (for phagocytosis experiments with paralyzed PMN and MF) by washing PMN six times by centrifugation at 200 × g. This procedure yielded a population of infected PMN in which the ratio of extracellular *L. major* promastigotes was <1 per 1000 PMN. PMN and supernatants were collected after 18 and 42 h of coinoculation for further analyses.

**Assessment of *L. major* survival in PMN**

The presence of viable intracellular *L. major* in PMN was visualized by performing Live/Dead (Molecular Probes, Leiden, The Netherlands) staining 18 and 42 h after infection of PMN. In addition, an end point titration in vitro culture (7) was used to quantify the number of viable parasites in the infected PMN population. In short, 5.0 × 10⁵ PMN were added in quadruplicate wells of 96-well microtiter plates containing biphasic NNN blood agar medium (50 μl of blood agar and 100 μl of complete RPMI 1640 medium and an end point titration with a dilution factor of 1.5 was conducted). The plates were then incubated at 27°C in a 5% CO₂ humidified atmosphere for 1 wk to allow the growth of *Leishmania*. The number of viable *Leishmania* per 1000 PMN was calculated from the last dilution where at least three of four wells had parasitic growth, considering the average plating efficiency of 10 promastigotes as determined for *L. major* previously (8).

**Electron microscopy and flow cytometry**

For structural preservation electron microscopy, cells were fixed with 5% glutaraldehyde for 1 h, treated with 1% OsO₄, for 2 h, and dehydrated in ethanol. The samples were embedded in Araldite (Fluka, Buchs, Switzerland). Ultrathin sections were contrasted with uranyl acetate and lead citrate and were examined with a Philips EM 400 electron microscope (Eindhoven, The Netherlands).

Labeling of apoptotic cells with Annexin V-FITC (Roche Molecular Biologicals, Mannheim, Germany) was performed as recommended by the manufacturer. Labeled cells were analyzed by flow cytometry using a FACSCalibur with CellQuest software (BD Biosciences, San Diego, CA).

**Cytokine measurement and chemotaxis**

MIP-1β concentrations in the supernatants were assessed by an ELISA kit (R&D Systems, Wiesbaden, Germany). Chemotaxis assays were performed with freshly isolated monocytes in 24-well Transwell plates (Costar, Leendon, Germany). Chemotaxis assays were performed as described for parasitized PMN and the number of viable *Leishmania* per 1000 PMN was calculated from the last dilution where at least three of four wells had parasitic growth, considering the average plating efficiency of 10 promastigotes as determined for *L. major* previously (9).

**Phagocytosis of infected PMN by autologous MF**

PMN and monocytes were isolated from buffy coat as described (6). PMN (1 × 10⁷/ml) were cocultivated with *L. major* at 37°C at a parasite to PMN ratio of 5:1. After 42 h of incubation, extracellular parasites were removed as described above. The number of parasites per PMN and the percentage of apoptotic PMN were assessed after staining with Syto-16 and Annexin V-Alexa 568 (Molecular Probes). On average, 70% of PMN were apoptotic (Annexin V-Alexa 568 positive) and >90% of PMN were infected with *L. major*. The infected cells harbored an average of 2.4 *Leishmania*. MF were generated by culturing autologous monocytes for 2 days in complete RPMI 1640 medium supplemented with 10 ng/ml M-CSF (Peprotech). MF were coincubated with infected PMN at 37°C at a parasite to macrophage ratio of 3:1.

**Results and Discussion**

Highly purified human PMN were coincubated with *L. major* promastigotes for 3 h. After removal of noningested parasites, the infected PMN were further cultured for 42 h. The majority of parasitized PMN became apoptotic and, consequently, phagocytosed by autologous MF. First, phagocytosis of unlabeled infected PMN was visualized 15 min after coinoculation using structural preservation electron microscopy. Second, phagocytosis of Syto-16/Annexin V-Alexa 568-stained infected PMN was assessed by confocal microscopy (LSM 510 META; Carl Zeiss, Jena, Germany).

**Assessment of survival and multiplication of *L. major* into MF**

The presence of viable intracellular *L. major* in MF was visualized after performing Live/Dead staining 18, 42, and 114 h after coinoculation with infected PMN. Supernatants were collected at these time points for cytokine assays. An end point titration was used to quantify the number of viable parasites in the macrophage cultures (7). In short, parasites were taken from 5 × 10⁵ MF after cell lysis in RPMI 1640 medium containing 0.01% SDS; an end-point titration was performed as described for parasitized PMN and the number of viable *Leishmania* per 1000 MF was calculated.

**Phagocytosis of infected apoptotic PMN by autologous MF**

PMN and monocytes were isolated from buffy coat as described (6). PMN (1 × 10⁷/ml) were cocultivated with *L. major* at 37°C at a parasite to PMN ratio of 5:1. After 42 h of incubation, extracellular parasites were removed as described above. The number of parasites per PMN and the percentage of apoptotic PMN were assessed after staining with Syto-16 and Annexin V-Alexa 568 (Molecular Probes). On average, 70% of PMN were apoptotic (Annexin V-Alexa 568 positive) and >90% of PMN were infected with *L. major*. The infected cells harbored an average of 2.4 *Leishmania*. MF were generated by culturing autologous monocytes for 2 days in complete RPMI 1640 medium supplemented with 10 ng/ml M-CSF (Peprotech). MF were coincubated with infected PMN at 37°C at a parasite to macrophage ratio of 3:1.

Two methods were applied to visualize the uptake of infected apoptotic PMN by MF. First, phagocytosis of unlabeled infected PMN was visualized 15 min after coinoculation using structural preservation electron microscopy. Second, phagocytosis of Syto-16/Annexin V-Alexa 568-stained infected PMN was assessed by confocal microscopy (LSM 510 META; Carl Zeiss, Jena, Germany).

**FIGURE 1.** Forty-two hours after infection apoptotic PMN contain intact and viable *L. major* promastigotes. *A*, Transmission electron micrograph of a PMN 42 h after infection with *L. major*. Arrows indicate intracellular *Leishmania* parasites and vacuolization around the nucleus of PMN (N), a sign of apoptosis (bar 9,000). High power magnification of *L. major* inside a parasitophorous vacuole (bar 25,000). Arrows indicate intact tubular structure. A typical 9:2 structure can be observed in the flagellum (F) and the flagellum pocket (P). Nuclear (N) and kinetoplast (K) structures appear intact (bar = 1 μm).
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Vacuolization around the nucleus (N) and condensed DNA indicate that the PMN are dying. The intracellular parasites showed perfectly intact ultra structure as evidenced by the clear microtubular profiles in the two sections of the flagellum and beneath the cell membrane of the body. For many intracellular Leishmania, the flagella (Fig. 1B, “F”) were not only seen in the flagella pocket (Fig. 1B, “P”) but two “cuts” of the flagellum were also evident. This observation indicates that the parasites did not lose their flagella inside PMN and thus had not transformed into amastigotes. This was supported by time-lapse documentation which showed active flagella movement of Leishmaniasis parasites inside PMN 42 h after infection (supplemental videos 1 and 2). 4 Using an end-point titration assay, we could demonstrate that the number of viable Leishmania remained stable inside PMN 18 and 42 h after infection (62.1 ± 3.1 and 59.3 ± 2.9 Leishmania per 1000 PMN, respectively, n = 4). Although these findings appear to contradict some earlier reports indicating that the PMN exert antileishmanial activity (10, 11), the data confirm other studies describing the disease-promoting effect of PMN in Leishmaniasis infection (12, 13). Importantly, our data are in line with the recent observation that apoptotic PMN exacerbate Leishmaniasis infection (14). Our findings suggest that although PMN do not kill L. major promastigotes, the parasites are not provided with a suitable environment for their multiplication inside PMN. Therefore, PMN can serve solely as temporary host cells for Leishmania.

Infected PMN produce MIP-1β and attract monocytes

Next, we addressed the question of whether infected PMN participate in the recruitment of MF to the site of infection. A screening of chemotactic proteins revealed that upon infection with L. major, PMN produced significant amounts of MIP-1β (Fig. 2A). MIP-1β is known to be chemotactic for monocytes (15). Using an in vitro migration assay, we found that supernatants taken from L. major-infected PMN indeed attracted monocytes (Fig. 2B). These data suggest that the secretion of chemokines such as MIP-1β by infected PMN participates in the recruitment of monocytes/MF to the site of infection.

MF internalize L. major-infected apoptotic PMN in vitro

After having shown that PMN can ingest Leishmania and that the parasites can survive in their promastigote form inside these cells for at least 42 h, even during the gradually occurring apoptosis of their host cells, we were interested in the fate of the parasites inside the dying PMN.

Apoptotic neutrophils are rapidly engulfed by MF (16). MF recognize apoptotic cells using specific receptors (17). To investigate whether MF ingested apoptotic PMN infected with intracellular Leishmania, they were coincubated with 42-h-old L. major-infected PMN in a calculated ratio of 3 intracellular parasites to 1 macrophage. The infected PMN were double-labeled with the nuclear stain Syto-16 to visualize the nuclei and kinetoplasts of Leishmania, and Annexin V-Alexa 568 to label apoptotic PMN (Fig. 3A). The double-labeled infected PMN were then coincubated with MF. Confocal microscopy revealed a rapid uptake of infected apoptotic PMN (Fig. 3B). Fifteen minutes after coincubation, Syto-16-stained parasites were observed in MF. The intact morphological appearance of nuclei and kinetoplasts of these intracellular parasites are strong indicators for their viability. A diffuse red staining was observed in these infected MF which represents the red Annexin V-Alexa 568 stain used to label apoptotic PMN (Fig. 3B).

These findings were strongly supported by electron microscopical analysis. A completely engulfed infected PMN in a macrophage phagosome can be seen in Fig. 3C. Inside the phagosome, both the condensed nucleus of the PMN and an intact parasitic structure are clearly visible. A time-lapse documentation demonstrates that the parasite remains inside the apoptotic PMN during engulfment by MF (supplemental video 3). These data demonstrate that MF take up infected apoptotic PMN and the parasite survives this initial phagocytosis process.

The intramacrophage viability of intracellular L. major parasites was assessed 18, 42, and 114 h after the phagocytosis of infected apoptotic PMN. Live/Dead staining revealed that the parasites not only survived but also multiplied inside MF (Fig. 4). Growth was first apparent 42 h after uptake and the number of intracellular parasites increased considerably during the 114 h observation period (Fig. 4).

Silent entry

Clearance of apoptotic cells is a major function of tissue MF. Uptake of apoptotic cells does not result in the activation of antimicrobial effector functions of the macrophage. Moreover, ingestion of apoptotic cells was reported to “silence” the functions of phagocytes in which the processing of anti-inflammatory mediator TGF-β has been reported to play a major role (16). The production of proinflammatory mediators such as IL-1β and TNF-α was reported to decrease after uptake of apoptotic cells (16). Interestingly, recent in vivo experiments demonstrated that uptake of apoptotic neutrophils exacerbated leishmanial infection in susceptible BALB/c mice. This disease-promoting effect was found to depend on TGF-β production by MF (14). In our study, uptake of L. major-infected apoptotic
PMN by MF induced the release of TGF-β (Fig. 5). The amount of secreted TGF-β following uptake of infected PMN was higher than that found after direct uptake of L. major promastigotes by MF (Fig. 5). No significant amounts of TNF-α were released (data not shown). These data suggest that uptake of infected apoptotic PMN can create an anti-inflammatory milieu, which is beneficial for Leishmania survival.

**Conclusions**

The presented data indicate that ingesting infected apoptotic PMN by MF is a way for Leishmania to enter into MF. This method of entry would promote parasite survival via at least two mechanisms. First, intracellular parasites in PMN have no direct physical interaction with macrophage surface receptors, and, consequently, the activation of MF does not occur. Second, the uptake of apoptotic cell silences the macrophage and no effector mechanisms are activated against the intracellular parasite. In summary, PMN are a perfect temporary shelter to preserve Leishmania from a hostile extracellular milieu before they enter their final host cells, the MF. Our data suggest a novel mechanism of infection of MF by L. major. The ability of PMN to phagocytose infected apoptotic PMN with condensed nucleus (N) and a structurally intact parasite (Lm) (bar = 1 μm, magnification ×6000).
the parasites to survive and maintain infectivity in PMN enables these organisms subsequently to establish productive infection in MF. These intracellular parasites can use granulocytes as “Trojan horses” to invade their definitive host cells, the MF.

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