FIGURE S1: Automatic image analysis procedure to monitor macrophage pyroptosis in *L. amazonensis*-infected BMDMs.

Live imaging was performed with the OPERA™ plate reader every 5 minutes following pyroptosis induction (ATP addition). Images were transferred to the Columbus Conductor™ Database and analyzed using the integrated Image analysis building blocks. (A) The sequential image segmentation process showing the identification and counting of Hoechst 33342-stained living/dead macrophages are shown for YO-PRO-1-stained dying cells. (B) The sequential image segmentation process showing the identification and counting of Hoechst-stained living/dead macrophages are shown for LTG-stained PVs.
FIGURE S2: LPS/ATP-treated *L. amazonensis*-infected macrophages die by pyroptosis (A) Representative image of *L. amazonensis* infected BMDMS after LPS stimulation and 4 hours of ATP stimulation obtained with the OPERA™ plate reader. A merge of the Hoechst 33342, mCherry, and bright field images is shown. The round swollen shape of both the cell body and the nucleus are typical for pyroptotic cells. Scale bar: 20 μm. (B) Detection of the release of cleaved caspase-1 in the supernatant (SN) from LPS and ATP-treated versus unstimulated (ctrl) infected BMDMs by Western blotting. Detection was performed 4 hours post ATP addition. (C) Evidence for IL-1 β secretion following ATP addition as detected by ELISA.
FIGURE S3: Characteristics of PVs and amastigotes during macrophage pyroptosis.

(A) representative field of infected macrophages at 3 time points corresponding to the 3 pyroptotic-related stages. The merged fluorescence and bright field images illustrate the behavior of LTG-stained PVs and mCherry-expressing amastigotes in Hoechst 33342-stained macrophages. Note the different location of amastigotes, either within PV (black arrow) or exposed to the outside milieu (white arrow) 240 min after ATP addition. Scale bar: 20 μm. (B) Monitoring of Individual PV area at 3 time points. Note the disappearance of some PVs at 240 min. (C) Relationship between the initial PV size and the extent of area change. The % of area change was recorded at 240 min for every single PV and classified in 3 categories depending on their initial size at 0 min. (D) Relationship between PV area reduction and parasite exposure for the 3 stages. (E-G) Scanning Electron Microscopy was performed on L. amazonensis (E, F) and L. donovani (G)-infected BMDMs. (E) Representative image of pyroptotic macrophages after 3 hours of ATP stimulation. Amastigotes are attached to dead macrophages and exposed to the extracellular milieu (red colorization). (F) Representative image of an untreated macrophages. Big globular cells correspond to heavily infected macrophages harboring large PVs. (G) Image of a representative pyroptotic L. donovani-infected BMDM. The exposition at the cell surface of L. donovani amastigotes (olive colorization, 1) is similar to that of L. amazonensis parasites and reveals the parasite flagellar pocket pointing towards the extracellular milieu (arrow, zoomed area, 2).
FIGURE S4: Schematic overview recapitulating the fate of intracellular *Leishmania* during macrophage pyroptosis.

The induction of pyroptosis in *Leishmania*-infected macrophages by ATP leads to two different scenarios. In both cases, PVs decay with a loss of LTG fluorescence, with or without reduction in area: In 61% of macrophages, a slow reduction of LTG staining occurs, with PV maintained *in cellula* and intracellular retention of virulent amastigotes (upper part). In 39% of macrophages, PVs decay faster and the inner part of the PV membrane is exposed on the macrophage surface (Exposure of the Luminal side of the parasitophorous Vacuole, ELV). ELV leads to the polarized exposure of virulent amastigotes that are maintained embedded in PV membranes (lower part). Parasite exposure occurs only during the first two stages of pyroptosis. The third stage is associated to more pronounced cellular alterations.
Movie 1: Real-time multiparametric analysis of *L. amazonensis*-infected macrophages during pyroptosis.

Pyroptosis was induced in *L. amazonensis*-infected BMDMs by sequential stimulation with LPS and ATP. Real-time analyses were performed to follow macrophage pyroptosis and amastigote localization during 240 minutes after ATP addition using the OPERA™ plate reader at 34°C, 7.5% CO₂. The movie was realized with sequential analyses of merged images for Ho 33342, LTG, mCherry fluorescence and TL pictures taken every 5 minutes.