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Addendum

Blocking autophagy to prevent parasite differentiation

A possible new strategy for fighting parasitic infections?

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The genome of *Trypanosoma cruzi* was surveyed for autophagy-related genes. We have identified all the essential genes except for the Atg12 conjugation system and demonstrated functionality of the putative *ATG4* and *ATG8* homologs. TcAtg4.1 was primarily involved in the proteolytic processing of TcAtg8.1, the *ATG8*-homolog that was found to be localized to autophagosomal membranes during starvation. Autophagy was also found to be strongly upregulated during differentiation between developmental stages, a process that is essential for the propagation of the parasite. Based on our work, new strategies for treatment of Chagas disease, a chronic debilitating condition still without suitable chemotherapy, can be envisioned.

Differentiation is a Key Process for *Trypanosoma cruzi* Infection

Trypanosomatids are a group of flagellated protozoan parasites that diverged from other eukaryotic organisms about 1800 million years ago, long before the appearance of red algae and fungi.[1] They are the etiological agents of endemic diseases prevalent mainly in developing countries. *Trypanosoma cruzi* causes Chagas disease, a chronic debilitating condition widespread in Latin America, *Trypanosoma brucei* takes its toll in the form of sleeping sickness in Africa, and *Leishmania* spp. cause different forms of leishmaniasis in America, Africa and Asia.[2] They are all heteroxenic parasites, meaning that their life cycle alternates between an insect vector and a mammalian host. Colonization of these very diverse environments by trypanosomatid parasites requires profound metabolic and morphological changes. The life cycle of *T. cruzi* involves four major developmental stages (Fig. 1). The parasite enters the mammalian host when the insect defecates in the vicinity of the bite and the natural infective stage, the metacyclic trypomastigote is carried into the wound by scratching, and then penetrates and infects nearby cells. Once inside the cell, metacyclic trypomastigotes differentiate into amastigotes. These replicative forms multiply in the cytoplasm and, after several rounds of replication, differentiate into trypomastigotes which gain access into the bloodstream and eventually invade new cells, thus perpetuating the infection. When the insect bites an infected mammal, the trypomastigotes carried over with the blood meal differentiate into epimastigotes, which are a replicative form living in the insect gut. In the rectum, where the insect´s urine is discharged, the epimastigotes differentiate to metacyclic trypomastigotes, which are able to start a new round of infection.[3] Differentiation appears to be triggered by the nutritional stress induced by the very low nutritional content of the insect's rectum.[4] The epimastigote stage, but not the other stages, contains an organelle called the reservosome,[5] which belongs to the lysosomal system and concentrates endocytosed proteins and lipids. The reservosomal content is consumed

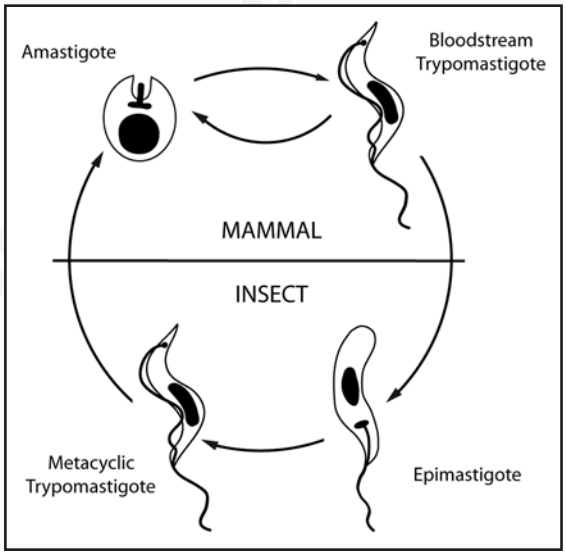

Figure 1. Life cycle of *Trypanosoma cruzi*.

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during differentiation to metacyclics, when the reservosomes shrink and finally disappear. The major cysteine proteinase of the parasite, cruzipain,[6] is highly concentrated and active in reservosomes and is supposed to be responsible for the massive proteolysis accompanying differentiation; cell-permeable inhibitors of the enzyme inhibit differentiation of epimastigotes to metacyclics,[7] and overexpression of the proteinase increases the rate of differentiation.[8]

T. cruzi Contains a Functional Autophagic System, which is Activated During Starvation, and Mediates Differentiation of the Parasite

In a recent paper[9] we demonstrated that *T. cruzi* contains a fully functional autophagic system, which was activated during starvation-induced stress and also during differentiation of the parasite. This work, together with some others,[10,11] demonstrates that autophagy is a very ancient process present already in protozoan parasites, thereby opening a completely new area in autophagy research. A very important question raised by these works is about the minimal system required for the normal functioning of autophagy. A bioinformatic survey for autophagy-related genes in the genomes of trypanosomatids namely revealed the presence of essential *ATG*-genes with the exception of the very important Atg12 conjugation system.[12,13] In *Leishmania* this notion remained somehow controversial,[10] however, the genome of *T. cruzi* seems to lack the *ATG5*, *ATG10* and *ATG12* genes, whereas a distant *ATG16* homolog might be present.[9] The functional role of the Atg12-Atg5 conjugate in autophagosome biogenesis is only now being recognized as an E3-like enzyme, necessary for Atg8-PE conjugation.[14] Enzymatic action in this step seems to be either not necessary in the very basic autophagic system in *T. cruzi* or perhaps carried out by other, unrelated proteins. A similar suggestion was also made for *Entamoeba sp.*, where no orthologues of these genes were found.[11]

In contrast to the Atg12-Atg5 system, genes involved in the Atg8-conjugation system, regulating membrane tethering and hemifusion during autophagosome formation,[15] were easily identified. However, whereas single *ATG4* and *ATG8* genes exist in yeast, several *ATG4* and *ATG8* homologs were found to be present in trypanosomatids with two of each present in *T. cruzi.*[9,16] The gene products showed functional conservation in their ability to complement yeast deletion strains (Δ*atg4* and Δ*atg8*). Both Atg4 variants, *TcATG4.1* and *TcATG4.2*, successfully replaced the yeast *ATG4* gene, whereas only one of the Atg8 variants, *TcATG8.1*, partially reconstituted autophagy in the *ATG8* deletion mutant. Only *TcATG4.1* very efficiently cleaved both Atg8 variants in vitro, suggesting that *TcATG4.*1 is the ortholog of yeast Atg4, whereas the function of *TcATG4.2* is, similarly to the function of several autophagins (Atg4) in human, more elusive.

Physiologically, autophagy is often triggered as a stress response, with starvation being the most common type of stress used in the experiments. In *T. cruzi*, starvation of epimastigotes also occurs naturally in the gut of the insect vector, which is known to suffer long periods of lack of food (up to 12 months). When *T. cruzi* epimastigotes were exposed to nutritional stress, autophagy was found to be induced,[9] thereby confirming the generality of the phenomenon. However, the studies demonstrated not only the presence of autophagosomes or related structures in the starved parasites (Figs. 2 and 3), but also confirmed that TcAtg8.1 (homologous to the human LC3), which is currently the best known biological marker for autophagy, is a suitable marker also in parasites. This is a further proof of a striking functional conservation of autophagy from protozoan parasites to human.

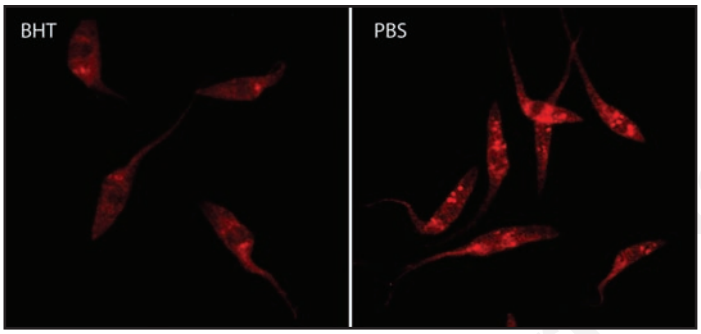

Figure 2. Sub-cellular localization of endogenous Atg8.1 in nutrient rich BHT medium (left panel) and after prolonged starvation in PBS (right panel) in untransfected *T. cruzi* epimastigotes was evaluated by indirect immunofluorescence experiments using anti-Atg8.1 specific antibodies and Alexa 546-conjugated secondary antibody.

Probably the most important finding of our article is that autophagy is also triggered during *T. cruzi* epimastigote to metacyclic trypomastigote spontaneous differentiation. A massive concentrating of TcAtg8.1 in autophagosomes was only observed in morphologically identifiable intermediate stages and not in normal epimastigotes nor in fully developed metacyclic trypomastigotes, a clear indication of a very dynamic process, which is only activated when needed. A critical role of autophagy in differentiation of protozoan parasites and other early-diverging eukaryotes is clearly emerging. Autophagy-mediated differentiation was thus observed not only in *T. cruzi*,[9] but also in *Leishmania sp.*[10,17] and *Entamoeba sp*.[11] Moreover, knocking-out Atg4 in *L. major* blocked differentiation of the parasite,[17] a process which seems critical for its infectiveness.

Conclusions

Despite of large number of infected people, adequate treatment for Chagas disease is not available. Chemotherapeutic agents currently in use are characterized by low efficiency and severe adverse effects. Therefore, there is a pressing need to develop new therapies. Inhibitors of cruzipain, the major lysosomal cysteine peptidase of *T. cruzi* were shown to effectively inhibit differentiation of the parasite,[7,18,19] however the mechanism of their action was not elucidated. Now we suggest that this is due to the inhibition of terminal turnover of proteins during the final step of autophagy-mediated differentiation. Several inhibitors of cruzipain have recently shown good results in animal models of infection, and have been reported to be in late-stage preclinical development.[20,21] Since it is highly likely that differentiation inside the human host is also mediated by autophagy, our results suggest that the Atg4 protease (autophagin) may be an alternative target, reasoning that targeting an upstream protease has several advantages.[22] Targeting human Atg4 by small synthetic inhibitors was recently suggested to be an attractive strategy for treatment of several conditions where autophagy is believed to be overly active.[23] Given the striking evolutionary conservation of the substrate specificity of this protease, these inhibitors, once approved for use in humans, could also be tried, not only for infections with *T. cruzi* and trypanosomatids but also for infections caused by other

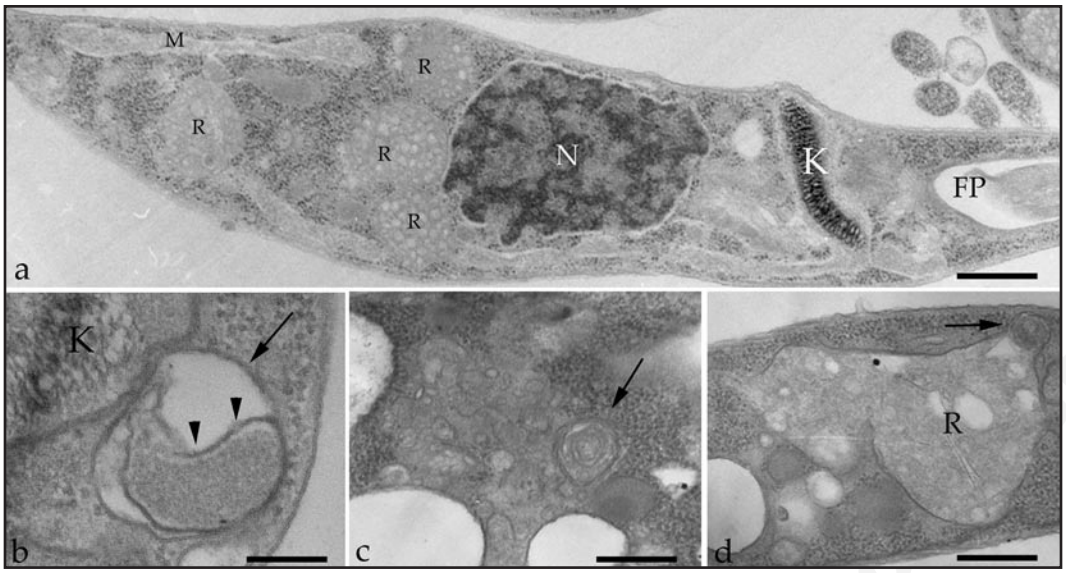

Figure 3. Transmission electron microscopy analysis of *T. cruzi* epimastigotes grown in rich medium (a) or starved in PBS for 16 hr (b–d). *N* nucleus, *K* kinetoplast, *R* reservosome, *M* mitochondria, *FP* flagellar pocket. In panel (b) external and internal membranes of an autophagosome are indicated by an arrow and an arrowhead, respectively. In (c) one myelin-like figure is depicted with an arrow and in (d) the arrow shows an electrondense material that resembles putative autophagic bodies inside reservosomes. *Scale bars* (a) 2 μm; (b) 0.25 μm; (c) 0.5 μm; (d) 1 μm.

eukaryotic pathogens, which depend on autophagy-mediated differentiation between various developmental stages.

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