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Processing and presentation of antigens derived from intracellular protozoan parasites

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Summary

Control of parasitic protozoan infections requires the generation of efficient innate and adaptive immune responses, and in most cases both CD8 and CD4 T cells are necessary for host survival. Since intracellular protozoa remodel the vacuolar compartments in which they reside, it is not obvious how their antigens enter the MHC class I and class II pathways. Studies using genetically engineered parasites have shown that host cell targeting, intracellular compartmentalization, subcellular localization of antigen within the parasite and mechanism of invasion are important factors determining the presentation pathway utilized. The recent identification of endogenous parasite-derived CD8 T cell epitopes have helped confirm these concepts as well as provided new information on the processing pathways and the impact of parasite-stage specific antigen expression on the repertoire of responding T cells stimulated by infection. Elucidating the mechanisms governing antigen processing and presentation of intracellular protozoa may provide important insights needed for the rational design of effective vaccines.

Introduction

Intracellular protozoan parasites represent a major cause of disease and despite years of effort, no effective vaccines have been developed for routine immunization against these pathogens. To succeed as parasites, these organisms need to achieve a fine balance with their hosts in order to establish chronic infections that promote transmission. Protozoan parasites have developed numerous strategies to avoid or manipulate host immune defenses [1–3]. While the intracellular life style adopted by major parasites such as *Leishmania spp, Toxoplasma gondii*, *Plasmodium spp* (the causative agent of malaria) and *Trypanosoma cruzi* provides protection against humoral attack, these pathogens must at the same time evade intracellular antimicrobial mechanisms. To do so, they often remodel the host cell compartments in which they reside.

T. cruzi, T. gondii and malaria parasites actively invade mammalian cells, while in contrast, *Leishmania* which lack an active invasion machinery are restricted to professional phagocytes, i.e. macrophages, neutrophils and dendritic cells (DCs). After phagocytic entry, *Leishmania*

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reside in phagosomes that fuse with late endocytic compartments [4]. Although they do not significantly remodel the phagosome, *Leishmania* amastigotes are adapted to survive and replicate within the hostile acidic environment of the mature phagolysosome. In the case of *T. cruzi*, while active invasion by trypomastigotes also leads to the formation of an acidic compartment, the parasite cannot survive the low pH of a lysosome-fused parasitophorous vacuole (PV) and rapidly escapes into the host cytosol [5]. A particularly interesting scenario is provided by *Toxoplasma gondii. T. gondii* tachyzoites actively infect host cells by a process involving the sequential discharge of parasite secretory organelles, leading to the formation of a highly specialized PV [6]. Actively remodel of the PV membrane (PVM), renders the PV incompetent for endosome/lysosome fusion and unable to acidify [7–8]. *T. gondii* tachyzoites rapidly multiply within the PV until parasite egress occurs followed by host cell lysis.

Because of the diverse life styles outlined above, processing and presentation of antigens of intracellular protozoa involves a set of distinct mechanisms that provide a fascinating perspective on this critical step in the induction of the immune response. This review highlights recent progress in the area comparing three examples of protozoa (*T. cruzi*, *T. gondii* and *Leishmania*) that dwell in different intracellular compartments.

Antigen presentation on MHC class I

Antigen subcellular localization

T. cruzi, *Leishmania* and *T. gondii* reside in distinct intracellular compartments, nevertheless, they all induce strong CD8 T cell responses. Ag localization within the parasite may be an important factor influencing this shared immunological activity. Pioneering studies showed that host cells infected with *T. cruzi* expressing secretory or GPI-anchored, but not cytosolic or transmembrane OVA, process and present peptides to CD8 T cells, thus suggesting that only released proteins gain access to the class I pathway [9]. A similar requirement was observed for *T. gondii* when transgenic tachyzoites expressing LacZ or OVA either in the cytosol or secreted into the PV were compared [10,11]. Likewise, in the case of *L. major*, OVA expressed as a secreted but not cytosolic Ag, efficiently triggers CD8 T priming both in vitro and in vivo [12].

Class I presentation pathways utilized by different protozoa

As *T. cruzi* resides in the host cytoplasm, it is not surprising that proteins released during normal cellular infection gain direct access to the classical cytosolic MHC class I pathway (Figure 1). This mechanism is supported by studies showing that mice lacking the transporter associated with Ag processing (TAP)-1 are highly susceptible to *T. cruzi* infection [13]. In contrast, the presentation pathways utilized by *Toxoplasma* and *Leishmania* that are sequestered inside vacuoles, are not so obvious. Since the Ags in question are synthesized by the parasite's own protein synthetic machinery without involving that of the host cell, we believe that it is appropriate to refer to this as "cross-presentation", a process utilized primarily by DC. Possible cross-presentation mechanisms employed include, i) phagocytosis of bystander-infected cells, ii) uptake of dead parasites and/or soluble material, iii) injection of Ag into the cytosol at the time of infection, iv) cross-presentation of Ags derived from PVs containing live parasites (Figure 1).

In the case of *L.major*, DCs infected with live promastigotes secreting OVA (*Lm*-NT-OVA) induce potent CD8 T cell responses, while DCs exposed to heat-killed (HK) *L. m*-NT-OVA are significantly less efficient [12]. The same study, ruled out a possible contribution of regurgitated peptides or proteolytically degraded soluble protein released by extracellular parasites or infected cells as a source of class I-binding peptides. It has been recently shown that during initial *Leishmania* infection, although some parasites are directly phagocytosed by

DCs [14•], neutrophils are the major host cell harboring live parasites and therefore, following apoptosis could be cross-presented by DCs [15••]. Furthermore, 1–2 weeks following *Leishmania* inoculation the majority of parasite-containing cells at the infection site expresses a phenotype compatible with that of DCs that have phagocytosed neutrophils [16••]. This is an important cross-presentation mechanism that deserves further attention.

Studies performed with the *Lm*-NT-OVA system, showed that cross-presentation of *L. major*-derived Ags by infected DCs is TAP independent both in vitro and in vivo, and is blocked by neutralization of phagosomal pH or by endosomal proteases inhibitors but it is not affected by suppression of proteasomal activity [17•]. A dispensable role for TAP was also observed when these experiments were extended to CD8 T cells induced by natural *Leishmania* Ags in vivo. Taken together, these findings strongly suggest that *L. major* Ags are processed via an intraphagosomal pathway. In contrast, a previous report suggested that presentation of the membrane gp46/M-2 glycoprotein by *L. amazonensis*-infected macrophages followed a cytosolic pathway [18]. Whether these discrepancies are due to the ability of different APCs to utilize distinct Ag processing mechanisms, or to intrinsic properties of *Leishmania* species remains to be elucidated.

In direct contrast to *Leishmania*, cross-presentation of OVA transgenic *T. gondii* (*Tg*-OVA) from different parasite strains is strictly TAP and proteasome dependent [11,17•,19•]. A TAP-dependent mechanism was also demonstrated for non-transgenic *T. gondii* Ags in vivo [20] and for the recently identified *T. gondii* GRA6 epitope HF10 [21••]. Moreover, HF10 processing also requires cytosolic proteasomal degradation and additional proteolysis by the ER aminopeptidase associated with antigen processing (ERAAP) [21••].

Given that *T. gondii* dwells in a unique PV sequestered from the host endocytic/exocytic compartments, one could hypothesize that cross-presentation occurs as a result of uptake of infected cells, dead parasites or soluble material released in the extracellular microenvironment. Nevertheless, multiple experimental approaches indicate that active invasion is required for cross-presentation of *T. gondii*-derived Ags [11,19•,22••]. Indeed, killed *Tg*-OVA parasites fail to induce CD8 T cell priming both in vitro and in vivo [22••]. It has also been shown that DCs exposed to soluble parasites products are unable to activate CD8 T cells [19•] and that parasite replication and host cell lysis is not required [19•,22••]. Similar results were observed with polyclonal CD8 T cells derived from mice infected with non-transgenic parasite [22••]. Furthermore, phagocytosis of live and/or antibody-opsonized parasites, which are targeted to conventional phagosomes [8][23], fails to induce CD8 T cell activation ruling out a possible contribution of phagocytic uptake of live parasites during infection [22••].

During invasion *T. gondii* secretes proteins into the host cell cytosol (reviewed in [24]) which could directly enter the endogenous class I pathway. Nevertheless, experiments blocking cell invasion indicated that such scenario is unlikely to occur [22••]. Moreover, the use of mixed haplotype cultures suggested that phagocytosis of infected cells does not contribute to cross-presentation of *T. gondii* Ags [[19•] and Goldszmid et al. unpublished]. More importantly in vivo transfer of infected MHC class I deficient cells also failed to induce CD8 T cell priming [22••].

The above findings suggest a pathway in which *T. gondii* proteins escape from the PV and are processed in the host cytosol. A previous report indicated that a dense granule-derived protein (GRA7) could be detected on the host cell surface, consistent with its transport from the PV [25]. To address this issue an elegant experiment was performed in which *T. gondii* expressing a Cre recombinase secreted into the PV (*Tg*-secCre), was used to infect host cells harboring a silent GFP gene that is activated upon Cre-mediated deletion of the transcriptional stop signal

[11]. GFP fluorescence was observed in *Tg*-secCre-infected cells but not in cells infected with parasites expressing a non-secreted Cre or incubated with supernatant from *Tg*-secCre cultures. These observations indicate that intact Cre recombinase can escape the PV and reach the host nucleus, thus supporting the hypothesis that after active infection, macromolecular parasite proteins secreted into the PV gain access to the host cytosol and enter the MHC class I pathway. Pores in the PVM have been previously described that allow exchange of small molecules < 1.3 kDa [26], and it was therefore unclear how large proteins can exit the PV. Live *T. gondii* is known to actively recruit host mitochondria and ER to the PV, presumably for nutrient acquisition [27]. Hence, we hypothesized that the intimate association of PVM and host ER (hER) could serve as a conduit for Ag escape into the host cytosol. Using immunogold labeling and in situ immunocytochemical staining for the luminal ER-specific marker glucose 6-phosphatase (G6Pase), an enzyme absent in *T. gondii* [28][29] we documented transfer of hER components into the PV indicating direct communication between the two compartments [22••]. In addition, we showed that *T. gondii* exploits the hER-associated degradation system to transport proteins into the cytosol, a process that has been implicated in cross-presentation of soluble and particulate-bound Ags in other systems [30–32]. Remarkably, in all the conditions assessed in which *T. gondii* was targeted to conventional phagosomes no ER recruitment or fusion was observed, nor was class I presentation activity detected arguing that active remodeling of the PV by the parasite is required for both processes [22••].

In vivo visualization of APC-CD8 T cell interactions

Two recently published studies using OVA-expressing fluorescent *T. gondii* and intravital two-photon microscopy have demonstrated that CD8 T cells engage in long lasting contacts with parasite-infected APCs within lymphoid organs [33••,34•]. Surprisingly, in both reports parasites could not be detected in a proportion of DCs associated with T cells. This observation was interpreted by Chtanova et al. [33••] as resulting from loss of fluorescence signal following parasite killing within the PV. In contrast, John at al. [34•] argued that these fluorescence-negative cells were never infected but did note a vacuolar phenotype that could be consistent with prior infection. This controversy highlights the need for further studies that more definitively determine whether APCs infection is critical for cross presentation of *T. gondii* Ags in vivo. In the above reports both macrophages and DCs were found to be the major APCs presenting to CD8 T cells in lymphoid tissue, and a prior study performed in the brains of chronically infected mice showed that CD8 T cells associate with infected-CD11b+myeloid cells but not with parasite-harboring astrocytes or neurons [35•].

Newly identified endogenous parasite epitopes

The sequencing of parasite genomes has provided new tools for screening and identification of natural occurring epitopes. Interestingly, in line with the data obtained with model Ags, in the case of both *T. cruzi* and *T. gondii* the newly identified immunodominant epitopes recognized by CD8 T cells correspond to secreted proteins [21••,36,37••]. Research performed on one of the recently characterized *T. gondii* epitopes has also provided new information on the role of ERAAP as noted above [21••], and established the importance of parasite-stage specific antigen expression in determining the repertoire of responding CD8 T cells. The latter conclusion was based on the observation that CD8 T cells specific for a GRA4 tachyzoite-derived epitope predominate during acute infection, whereas CD8 T cells recognizing ROP7 (a protein expressed on both tachyzoites and bradyzoites) are triggered primarily during chronic infection [37••].

Immunity-related GTPases (IRG) and antigen presentation

The IRG proteins are a family of interferon-induced proteins involved in resistance to intracellular pathogens [38]. IRG-mediated resistance to *T. gondii* depends on the recruitment

of IRGs to the PVM and subsequent vacuolar disruption and parasite killing, which has been argued to involve either autophagy or necrotic cell death [39–42]. Interestingly, a member of the IRG family, Irgm3, has recently been implicated in cross-presentation of OVA-coated latex beads by DCs [43••]. Nevertheless, *T. gondii*-infected DCs deficient in either Irgm3 or its relative Irgm1, do not display any noticeable defects in CD8 T cells activation [[19•] and Goldszmid et al. unpublished]. The suggested role of Irgm3 in cross-presentation of bead-associated Ags stems from its negative effects on phagosomal maturation. Since *T. gondii* does not reside within a phagosome this could explain the difference observed in the outcome of Irgm3 deficiency in the two systems. Interestingly, it was also found that IFN-γ activated wild type but not Irgm3 deficient *T. gondii*-infected macrophages cross-present Ag to CD8 T cells [19•]. Whether this difference between DCs and macrophages is due to uncontrolled parasite growth and death of the infected Irgm3 deficient macrophages or reflects different roles for IRGs in macrophages versus DCs was not investigated.

Antigen presentation on MHC class II

That uptake of dead parasites or parasite-derived material leads to class II presentation seems intuitive. Nevertheless, there is evidence that infected APCs present Ag to CD4 T cells, and that in common with MHC-I presentation, Ag compartmentalization may influence this process. In the case of *Leishmania*, Ags released into the phagosome could be readily processed and presented on MHC-II molecules (Figure 2). In this respect, OVA expressed on the plasma membrane of *L. major* was presented to naïve CD4 T cells in vivo, while presentation of cytosolic OVA occurred only at high-dose infection, albeit with a significantly lower efficiency [44]. Nevertheless, it is well established that the immunodominant epitope recognized by CD4 T cells in *L. major*-infected BALB/c derives from the cytosolic protein LACK [45]. Interestingly, the processing of this epitope requires the presence of DM, a non-classical MHC-II molecule with peptide editor functions [46].

In the case of *T. gondii*, MHC-II presentation has been shown to be restricted to secreted proteins [47]. However, in contrast to MHC-I presentation, active invasion is not required and activation of CD4 T cells is more efficient after parasite phagocytosis [22••]. The lower efficiency observed with actively infected DCs may stem from down modulation of MHC-II molecules induced by *T. gondii* [48,49] or simply reflect the low level phagocytic uptake of tachyzoites or parasite-secreted proteins that occurs during active infection (Figure 2).

Conclusions

An important lesson from the studies described in this review is that concepts derived from experiments using inert model cargo do not necessarily translate to live intracellular pathogens such as the protozoan parasites discussed here. A further lesson is that different parasite species and/or developmental stages may employ mechanisms that are quite distinct. While much of the current information stems from studies on organisms expressing model Ags, the advent of new technologies for identifying naturally occurring T cell epitopes has now initiated a new era in which processing and presentation pathways can be studied in a more physiological setting. The information gained should be more directly applicable to candidate vaccines and the elucidation of the requirements for their efficacy. Regardless, these studies will continue to offer major insights into the basic cell biology of the host-pathogen interaction as it pertains to immune function.

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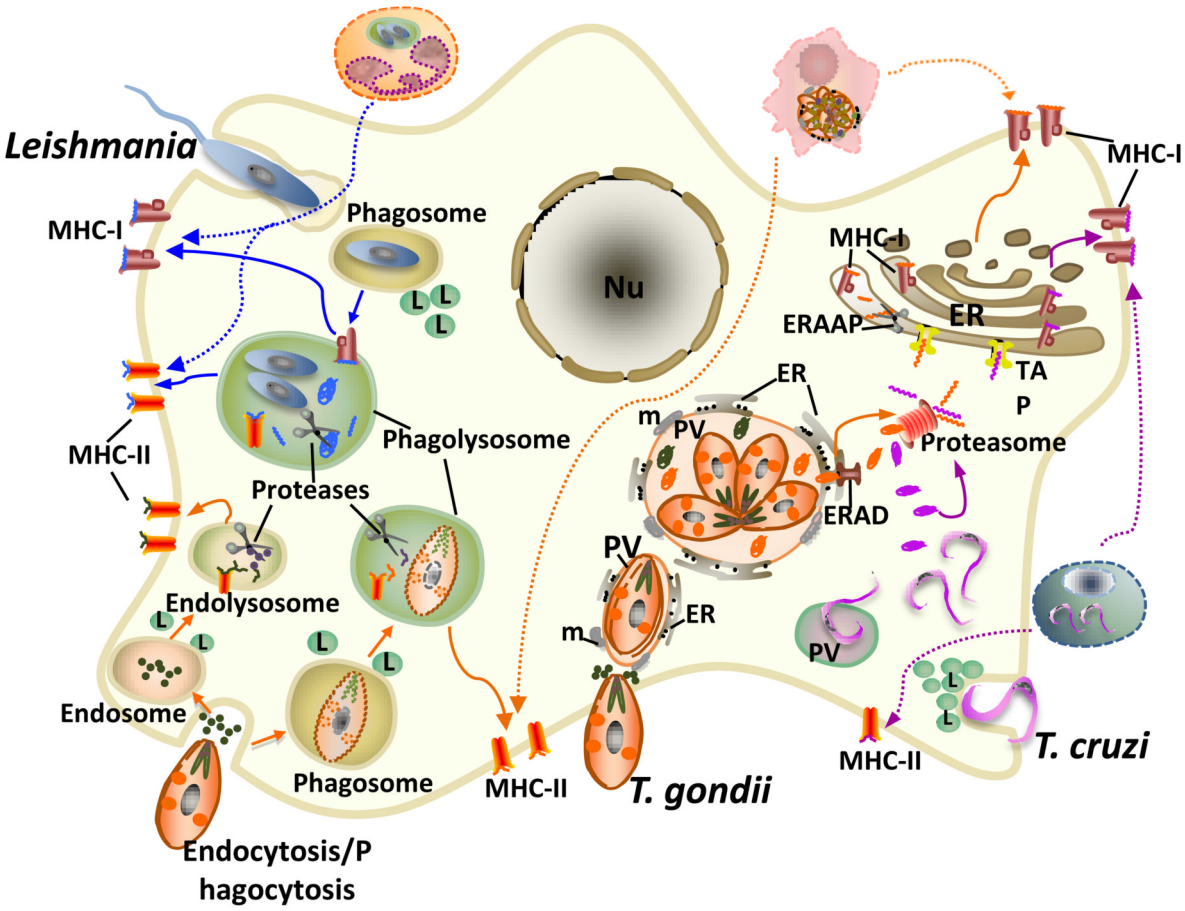

Figure 1. Working models for MHC-I and MHC-II-restricted presentation of Ags derived from *Leishmania*, *T. gondii* and *T. cruzi* parasites

Leishmania (blue arrows) are taken up by phagocytosis, and replicate within a phagolysosome. Ag processing occurs within the phagolysosome where secreted/released parasite proteins are degraded by endosomal proteases and the resulting peptides loaded onto MHC-I and MHC-II molecules followed by transport of the MHC-peptide complexes from the phagolysosome to the cell surface. In the case of *T. gondii* (orange arrows), MHC-I processing occurs after active invasion and fusion of the resulting PV with the host ER. Parasite proteins secreted into the PV lumen are retrotranslocated by the ER-associated degradation system (ERAD) to the cytosol where they are degraded by the proteasome and the resulting peptides transported via TAP into the ER for additional proteolysis by ERAAP and loading onto MHC-I molecules. No MHC-I processing results from phagocytic uptake of live or dead parasites or from proteins injected into the cytosol during invasion. For MHC-II processing, *T. gondii*-derived Ags can be obtained from phagocytosis or endocytosis of whole tachyzoites or parasite-derived products and it is unclear whether proteins derived from the PV can also access the MHC-II pathway. *T. cruzi* (purple arrows) invasion induces the recruitment and fusion of lysosomes to the plasma membrane and results in an acidic PV where it resides transiently before escaping into the host cell cytosol. Processing of parasite-released proteins follows the cytosolic proteasome/TAP-dependent pathway. Dotted arrows indicate the potential contribution of uptake of infected cells for both MHC-I and MHC-II processing. Nu, nucleus; m, mitochondria; L, lysosome.