H-2M molecules, like MHC class II molecules, are targeted to parasitophorous vacuoles of *Leishmania*-infected macrophages and internalized by amastigotes of *L. amazonensis* and *L. mexicana*

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SUMMARY

In their amastigote stage, *Leishmania* are obligatory intracellular parasites of mammalian macrophages, residing and multiplying within phagolysosomal compartments called parasitophorous vacuoles (PV). These organelles have properties similar to those described for the MHC class II compartments of antigen-presenting cells, sites where peptide-class II molecule complexes are formed before their expression at the cell surface. After infection with *Leishmania amazonensis* or *L. mexicana*, endocytosis and degradation of class II molecules by intracellular amastigotes have also been described, suggesting that these parasites have evolved mechanisms to escape the potentially hazardous antigen-presentation process. To determine whether these events extend to other molecules of the antigen-presentation machinery, we have now studied the fate of the MHC molecule H-2M in mouse macrophages infected with *Leishmania* amastigotes. At least for certain class II alleles, H-2M is an essential cofactor, which catalyses the release of the invariant chain-derived CLIP peptide from the peptide-binding groove of class II molecules and facilitates the binding of antigenic peptides. H-2M was detected in PV of mouse macrophages infected with various *Leishmania* species including *L. amazonensis*, *L. mexicana*, *L. major* and *L. donovani*. PV thus contain all the molecules required for the formation of peptide-class II molecule complexes and especially of complexes with parasite peptides. The present data indicate, however, that if this process occurs, it does not lead to a clear increase of SDS-stable compact αβ dimers of class II. In PV that contained *L. amazonensis* or *L. mexicana*, both class II and H-2M molecules often colocalized at the level where amastigotes bind to the PV membrane, suggesting that these molecules are physically associated, directly or indirectly, and possibly interact with parasite components. Furthermore, as class II molecules, H-2M molecules were internalized by amastigotes of these *Leishmania* species and reached parasite compartments that also contained class II molecules. Immunostaining of H-2M within parasites was increased by treatment of infected macrophages with the cysteine protease inhibitors Z-Phe-AlaCHN₂ or Z-Phe-PheCHN₂ or by incubation of the parasites with the same inhibitors before infection. These data thus support the idea that amastigotes of certain *Leishmania* species capture and degrade some of the molecules required for antigen presentation. To examine whether endocytosis of class II molecules by the parasites occurs through interactions with parasite components involving their peptide-binding groove, we made use of the fact that a large fraction of the class II molecules of H-2Mα knock-out H-2β mice are occupied by the peptide CLIP and are unable to bind other peptides. We found that, in *Leishmania*-infected macrophages of these mutant mice, class II-CLIP complexes reached PV and were internalized by amastigotes. These results thus prove that endocytosis of class II molecules by amastigotes (1) is H-2M-independent and (2) does not necessarily involve the peptide-binding pocket of these molecules. Altogether, these data are compatible with an endocytic mechanism based on general properties shared by classical and non-classical class II molecules.

Key words: Macrophage, *Leishmania* spp., Parasitophorous vacuole, MHC class II molecule, H-2M molecule, Invariant chain, Internalization

INTRODUCTION

The protozoan parasites *Leishmania* are the etiological agents of a large spectrum of diseases in humans, ranging from relatively mild cutaneous lesions to visceral infections, which are fatal if left untreated. These pathogens alternate between two main stages, a flagellated promastigote form, which lives as an extracellular parasite of the digestive tract of hematophagous insect vectors, and a non-motile amastigote form, which lives as an obligatory intracellular parasite of mammals. Macrophages are the mammalian host cells where amastigotes survive and multiply, within membrane-bound
compartments called parasitophorous vacuoles (PV). Several studies have shown that, after phagocytosis, these organelles are formed by phagosome maturation and fusion with late endosomes and lysosomes. Consequently, they share properties with these late endocytic compartments, namely an acidic internal pH, the presence in their lumen of acid hydrolases including proteases, and the presence in their membrane of lysosomal glycoproteins and of specific small GTP-binding proteins (for a review, see Antoine et al., 1998). In antigen-presenting cells (APC), like dendritic leukocytes, macrophages and B lymphocytes, late endocytic compartments intersect newly synthesized MHC class II molecules on the way towards the cell surface. For this reason, they are also called MIIC, for MHC class II compartments (Kleijmeer et al., 1997). Class II molecules are targeted to these organelles, owing to their transient association with the invariant chains Ii, a portion of which, called CLIP (class II-associated Ii peptide), also blocks the peptide-binding groove of class II molecules (reviewed in Germain et al., 1996). MIIC appear to be the major sites where peptide-MHC class II molecule complexes, which constitute ligands for CD4+ T lymphocytes, are generated. For this process to occur, Ii must be degraded and the class II peptide-binding groove freed of CLIP. The release of the peptide CLIP is catalysed by a newly discovered MHC molecule, called H-2M in mice and HLA-DM in humans. This molecule has also been described as favouring the formation of high stability peptide-class II molecule complexes (for reviews, see Roche, 2005; Kropshofer et al., 1997b).

Like MIIC, PV of Leishmania-infected macrophages activated with IFN-γ were found to contain MHC class II molecules apparently devoid of Ii chains in their limiting membrane (Antoine et al., 1991; Lang et al., 1994a,b). However, in the presence of certain protease inhibitors, PV-associated class II molecules remain complexed with Ii chains or Ii chain fragments, suggesting that class II reaching this compartment is neo-synthesized and that Ii chains are normally degraded in PV (De Souza Leao et al., 1995). Taken together, these observations and earlier findings concerning the PV origin and properties were consistent with these organelles being potential sites for the formation of complexes between parasite peptides and class II molecules, and with infected macrophages being potential APC for Leishmania-specific CD4+ T lymphocytes. However, formation of such complexes in PV remains to be documented. On the other hand, several findings suggest that parasites have evolved mechanisms to thwart the antigen presentation process. Thus, with some Leishmania species (L. amazonensis, L. mexicana), PV-associated class II molecules are preferentially located at the attachment zone of amastigotes to the PV membrane, which could reflect a sequestration of these molecules at this level (Lang et al., 1994a; Antoine et al., 1998). Furthermore, the same Leishmania species internalize and very likely degrade at least some of the class II molecules that reach PV. These phenomena were not observed with other proteins of the PV membrane like lamp1, lamp2, macrosialin, rab7p (De Souza Leao et al., 1995) and the antigen (Ag) recognized by the MOMA-2 antibody (Ab) (J.-C. Antoine, unpublished results).

To explore whether these events affect other molecules of the Ag presentation machinery, we have now studied the fate of H-2M molecules in Leishmania-infected macrophages. The interest for these molecules in leishmaniasis was recently strengthened by results showing that they are key elements in the control of L. major infections (Swier et al., 1998). Using macrophages of H-2Mα knockout H-2b mice, we have also asked whether the internalization of MHC class II molecules by amastigotes was dependent upon the presence of H-2M molecules.

**MATERIALS AND METHODS**

**Mice**

Female BALB/c, C57BL/6 and Swiss nu/nu mice aged 1-4 months were obtained from the Pasteur Institute (Paris, France) or from Iffa Credo (St Germain-sur-l’Arbresle, France). Female Syrian hamsters were obtained from the Centre d’Elevage des Animaux de Laboratoire (Ardenay-sur-Mérize, France). 6- to 10-month-old female H-2Mα knockout mice on a mixed B6x129 genetic background (Miyazaki et al., 1996) were kindly given by Drs C. Benoist and D. Mathis (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France).

**Parasites**

Leishmania amazonensis strain LV79 (MPRO/BR/72/M1841) and strain PH8 (IFLA/BR/67/PH8), L. mexicana strain M379 (MNYC/BZ/62/M379) and L. major strain NIH173 (MHOM/IR/-173) were kept virulent by passage in BALB/c or nu/nu mice. L. Donovan strain LV9 (MHOM/ET/67/Hu3:LV9) was maintained virulent by passage in hamsters. Amastigotes were prepared from mouse cutaneous lesions (L. amazonensis, L. mexicana, L. major) or from hamster spleens (L. donovani) as described (Antoine et al., 1989; Channon et al., 1984).

**Infection of macrophages in cultures**

Preparation of mouse bone marrow-derived macrophages and infection with amastigotes were performed as described (Antoine et al., 1991; De Souza Leao et al., 1995). In brief, bone marrow cells were allowed to differentiate into macrophages on 12 mm round glass coverslips for light microscopic studies and in 100 mm tissue culture dishes (Tanner, Trasadingen, Switzerland) for biochemical assays. Cells were cultured in RPMI 1640 medium (Seromed, Berlin, Germany) supplemented with 10% foetal calf serum (FCS), Dutscher, Brumath, France), 50 i.u./ml penicillin (Seromed), 50 μg/ml streptomycin (Seromed) and 10-15% L-929 fibroblast-conditioned medium. After 5 days at 37°C, adherent cells were washed with Dulbecco’s phosphate-buffered saline (PBS) and cultured for a further 24 hours in culture medium with or without 2.5% conditioned medium. Macrophages were then infected at an amastigote-to-host cell ratio of 4:1 or 5:1 and incubated for various time periods at 34°C (infections with L. amazonensis, L. mexicana or L. major) or 37°C (infections with L. donovani). Before or after infection, macrophages were treated with IFN-γ (10 to 25 U/ml, Genentech, Inc., San Francisco, CA, USA) or left with control medium. Uninfected cultures, similarly treated, were run in parallel. In some experiments, uninfected and infected macrophages were incubated for 20 hours (from 28 to 48 hours after infection) with 5 μM of the cytotoxic protamine inhibitors N-benzoxycarbonyl-phenylalanin-4-4-phenylalanin (Z-Phe-AlaCHN2) or N-benzoxycarbonyl-phenylalanin-4-4-phenylalanin (Z-Phe-PheCHN2) or with 0.2% DMSO, a concentration of solvent similar to that used in cultures treated with the inhibitors. Alternatively, amastigotes were incubated for 15 hours with 5 μM of the above inhibitors or with 0.2% DMSO in RPMI 1640, 20 mM Hepes, 10% FCS and penicillin/streptomycin or in Schneider's medium (Gibco, Paisley, UK), pH 5.3, containing 10% FCS and 20 μg/ml chloramphenicol (Coopération Pharmaceutique Française, Melun, France). Amastigotes were then washed and used to infect the macrophages.
which, 24 hours before, had been incubated with IFN-γ or control medium.

**Antibodies**

The monoclonal Ab (mAb) M5/114 (rat IgG2b) specific for I-Ab<sup>bdq</sup> and I-E<sup>dd</sup> (Bhattacharya et al., 1981), and the mAb Y-3P (mouse IgG2a) specific for I-A<sup>b</sup> (Janeway et al., 1984), were used as hybridoma culture supernatants or purified as described (Lang et al., 1994a). The purified mAb 30-2 (mouse IgG1) specific for CLIP peptide-I-A<sup>b</sup> complexes (Eastman et al., 1996) was kindly provided by Dr A. Rudensky (University of Washington School of Medicine, Seattle, USA). The mAb In-1 (rat IgG2b), specific for invariant chain (Ii) (Koch et al., 1982), was obtained from Dr N. Koch (Institut für Immunologie und Genetik, Heidelberg, Germany) and used as hybridoma supernatant. The mAb 2A3-26, a mouse IgG1 recognizing a plasma membrane antigen of *L. amazonensis* amastigotes, was purified as described (Lang et al., 1994a). Biotinylation of this antibody was done using D-biotinyl-e-amidocaprylic acid-N-hydroxysuccinimide ester (Pierce, Rockford, IL, USA), according to the manufacturer’s protocol. Rabbit immune sera specific for the cytosolic tails of I-A<sup>d</sup> (YRSGGTSRHPGPL), I-E<sup>a</sup> (YKGKKRNVVERRQGAL) and H-2M<sup>b</sup> (KSHSSSYTPlS-GSTYPEGRH) were generously provided by Drs C. Bonnerot and S. Amigorena (Institut Curie, Paris, France). They were raised against peptides coupled to keyhole limpet hemocyanin. Rabbit anti-H-2M<sup>b</sup> Ab was affinity purified from the anti-H-2M<sup>b</sup> cytosolic tail immune serum by chromatography through a HiTrap column (Pharmacia, Uppsala, Sweden) containing immobilized peptide directly coupled to the beads. The mAb LO-DNP-57 (rat IgG2b), specific for dinitrophenol (Caltag, San Francisco, CA, USA), the mAbs A10.3.2 and G21.10 (mouse IgG1), specific for serotonin (a drug, such as 5-bromo-4-chloro-3-indolyl phosphate and Nitroblue Tетразолиум as substrates (Sigma)).

**Preparation of macrophage lysates and suspensions**

48 hours after infection, macrophages cultured in 100 mm tissue culture dishes were washed once with PBS and then lysed with either Chaps lysis buffer (20 mM Tris-HCl buffer, pH 7.4, containing 130 mM NaCl and 1% w/v Chaps) or 1% Chaps lysis buffer (20 mM Tris-HCl buffer, pH 7.4, containing 130 mM NaCl and 1% w/v Chaps) or 1% dimethylammonio-1-propane sulfonate, Sigma Chemical Co., St Louis, MO, USA) or NP-40 lysis buffer (50 mM Tris-HCl buffer, pH 7.4, containing 25 mM KCl, 5 mM MgCl<sub>2</sub> and 1% Nonidet P-40 (Sigma)). A cocktail of protease inhibitors was added in lysis buffers as described earlier (De Souza Leao et al., 1995). After 30 minutes on ice (Chaps lysis buffer) or 10 minutes at room temperature and 20 minutes on ice (NP-40 lysis buffer), a sample was withdrawn for macrophage nucleus counting and lysates were centrifuged at 1,000 g for 10 minutes at 4°C. Supernatants were recovered and processed immediately for SDS-PAGE or stored at −70°C until use.

Alternatively, after washing with Dulbecco’s PBS, macrophages in tissue culture dishes were recovered by scraping in 0.15 M NaCl containing protease inhibitors. Cell suspensions were immediately frozen after macrophage nucleus counting or processed for SDS-PAGE. For each experiment, lysates or suspensions of uninfected macrophages were prepared in parallel and used as controls.

**SDS-PAGE and western blot analysis**

Macrophase suspensions or lysates were diluted with 1.5-fold concentrated Laemmli’s sample buffer in the presence or absence of 2-mercaptoethanol (Laemmli, 1970). After 20-30 minutes at room temperature or 5 minutes at 100°C, samples were electrophoresed on 10% or 12% SDS-acrylamide gels and then electrotransferred to Immobilon-P membrane (Millipore, Bedford, MA, USA) or to Hybond-C Extra nitrocellulose membrane (Amersham, Buckinghamshire, UK). Blots were then successively incubated with primary Ab and adequate alkaline phosphatase conjugates. Protein bands were visualized using 5-bromo-4-chloro-3-indolyl phosphate and Nitroblue Tetrozolium as substrates (Sigma).

**Immunofluorescence microscopy**

Single immunofluorescence labelling of macrophages on coverslips, as well as double staining with primary antibody belonging to different species, were done 24-72 hours after infection as detailed previously (Lang et al., 1994a). As controls, uninfected macrophages cultured in parallel were similarly processed. Briefly, cells were fixed for 1 hour at room temperature with 4% paraformaldehyde in 0.1 M sodium cacodylate, HCl buffer, pH 7.4, quenched with 50 mM NH<sub>4</sub>Cl in PBS and permeabilized with 0.01% saponin (Sigma) in PBS containing 10% serum of the same species as fluorochrome-conjugated Ab(s). Coverslips were then deposited upside down on parafilm in a 30 μl drop of primary Ab(s). After three washes with PBS containing 0.01% saponin, coverslips were incubated as above with fluorochrome conjugates and washed again.

Double immunofluorescence labellings with the anti-amastigote mAb 2A3-26 and either the mouse anti-I-A<sup>b</sup> mAb Y-3P or the mouse anti-I-A<sup>b</sup>-CLIP mAb 30-2 were performed as follows. Fixed and permeabilized macrophages were first incubated with Y-3P or 30-2 followed by an anti-mouse Ig fluorescein conjugate, as described above, after which they were post-fixed for 15 minutes with paraformaldehyde, quenched with NH<sub>4</sub>Cl and again successively incubated with mouse IgG (Jackson Laboratories, West Grove, PA; 100 μg/ml in PBS-saponin), biotinylated 2A3-26 (10 μg/ml in PBS-saponin containing 100 μg/ml mouse IgG and 0.25% gelatin) and Texas Red-conjugated streptavidin (Sigma; 5 μg/ml in PBS-saponin containing 0.25% gelatin).

In some experiments, cell preparations were incubated for 5 minutes with 7.5 μg/ml propidium iodide (Aldrich, Milwaukee, Wisconsin, USA) in PBS to stain nuclei of host cells and parasites. Cells were counted in Mowiol 4-88 (Calbiochem., San Diego, CA, USA) and then viewed with a Zeiss axiophot conventional fluorescence microscope (Oberkochen, Germany) or with a Leitz confocal laser scanning microscope (Wild Leitz Instruments, Heidelberg, Germany), which used an argon-krypton laser operating in multi-line mode (Lang et al., 1994a).

**RESULTS**

**Expression of H-2M molecules in infected and uninfected macrophages**

The percentage of bone marrow-derived macrophages expressing H-2M in various experimental conditions was assessed by immunofluorescence microscopy. For these experiments, polyclonal antibodies specific for the cytoplasmic tail of H-2M<sub>b</sub> were used. As shown in Fig. 1A, expression of H-2M<sub>b</sub> in this cell type was highly dependent upon IFN-γ activation and occurred even after its infection with amastigotes of *L. amazonensis*. 60-80% of cells were generally found positive for H-2M<sub>b</sub> whereas in the same preparations, 80-90% of cells expressed MHC class II molecules. To demonstrate the specificity of the staining, polyclonal antibodies were preincubated with the cytosolic peptide of H-2M<sub>b</sub> or with the irrelevant cl (12-26) peptide (cl, phase λ repressor). The H-2M<sub>b</sub> peptide but not the irrelevant cl peptide completely blocked the immunofluorescent signal (Fig. 1A).

**Subcellular localization of H-2M molecules in infected and uninfected macrophages**

We investigated the subcellular localization of H-2M using conventional immunofluorescence microscopy and confocal
Expression of H-2M in uninfected macrophages or macrophages infected with *L. amazonensis* amastigotes. Macrophages from BALB/c mice were infected with a 4:5:1 parasite:host cell ratio. IFN-γ (25 U/ml) was added to some wells 8 and 32 hours after infection (A) or 24 hours before infection and at the time of parasite addition (B). At 48 hours post-infection, macrophages were fixed and processed for immunofluorescence staining. Uninfected macrophages processed in a similar manner were run in parallel. (A) Percentage of H-2M-positive macrophages in cultures submitted to various experimental conditions and specificity of H-2M staining. Macrophages were labelled with a rabbit anti-H-2Mβ immune serum (diluted 1/1000) and an appropriate fluorochrome conjugate. To test the specificity of the stainings, immune serum dilutions were pre-incubated for 3 hours at 4°C with the H-2Mβ (224-243) cytosolic peptide (1.4 μg/ml) used for immunizations or with the same concentration of the irrelevant peptide cl (12-26) before contact with the cell preparations. At least 500 macrophages per cell preparation were counted. (B) Percentage of class II- or/and H-2Mβ-positive PV in infected macrophages treated with IFN-γ. Cells were double stained with the anti-MHC class II molecule mAb MS5/114 and with the anti-H-2Mβ immune serum followed by appropriate Texas Red and fluorescein conjugates. For some coverslips, the Ab mixture was pre-incubated for 3 hours at 4°C with the H-2Mβ (224-243) cytosolic peptide (14 μg/ml) before contact with the cell preparations. At least 500 PV per cell preparation were counted. The data presented are representative of two similar experiments.

*Fig. 1.* Expression of H-2M in uninfected macrophages or macrophages infected with *L. amazonensis* amastigotes. Microscopy. In contrast to MHC class II molecules, which were present both on the plasma membrane and in internal vesicles of uninfected macrophages, H-2M could be detected solely in intracellular compartments. H-2M-containing vesicles were mainly confined to the perinuclear area and many of them contained for MHC class II molecules (Fig. 2A-C). After infection, H-2M could be localized on the PV membrane, and the staining at this level was blocked by the H-2Mβ cytosolic peptide, confirming its specificity (Fig. 1B). All the H-2M-containing PV were also class II-positive, as indicated in Fig. 1B for macrophages infected with amastigotes of *L. amazonensis*. However, the percentage of H-2M-positive PV was lower than the percentage of class II-positive PV, a result very likely due to the weaker immunofluorescent signal obtained for H-2M. In macrophages infected with *L. amazonensis* or *L. mexicana*, a strong depletion of the H-2M-positive vesicles as detected in uninfected macrophages was also noted, suggesting that these vesicles fuse with PV and participate in the formation of these huge compartments. The same phenomenon could be observed in *L. donovani*-infected macrophages, in which case the depletion correlated with the number of small individual PV. In contrast, a different pattern was found in macrophages infected with *L. major*, since PV harbouring this species were generally faintly labelled with anti-H-2M and no depletion of H-2M-containing vesicles was noted.

The distribution pattern of PV-associated H-2M molecules was also dependent upon the *Leishmania* species harboured, as previously seen for PV-associated classical class II molecules (Antoine et al., 1991; Lang et al., 1994a,b). Thus, in the large communal PV that contained *L. amazonensis* or *L. mexicana*, H-2M molecules were located mainly in a restricted area also enriched in class II molecules and corresponding to the amastigote-binding sites (Fig. 2D-F, Fig. 3A). In contrast, in the individual PV that harboured *L. major*, H-2M molecules were always homogeneously distributed as were the class II molecules (Fig. 3B). Finally, in *L. donovani*-containing PV, a more complex pattern was obtained for H-2M distribution, since the staining was homogeneous or restricted to a pole of the PV (Fig. 3C).

**Internalization of H-2M by amastigotes of *L. amazonensis* and *L. mexicana***

To determine whether the internalization of classical class II molecules by intracellular amastigotes of *L. amazonensis* and of *L. mexicana* (De Souza Leao et al., 1995; Antoine et al., 1998) is accompanied by H-2M internalization, infected macrophages were fixed, stained for class II and H-2M and the intra-parasite structures containing class II molecules examined for the presence of H-2M molecules by confocal microscopy. The results in Fig. 4A-C show that, in addition to classical class II molecules, H-2M molecules could be detected in internal vesicles of amastigotes, and furthermore that both molecules generally colocalized in the same structures. These vesicles were mainly located in the posterior pole of the parasites, just below the portion of PV membrane where PV-associated class II and H-2M molecules were often concentrated. To analyse whether the H-2M-containing vesicles correspond to the parasite lysosomal compartments also known as megasomes and previously described as sites of degradation of internalized classical class II molecules (De Souza Leao et al., 1995), infected macrophages were treated with the irreversible cysteine protease inhibitor Z-Phe-AlaCHN₂. This compound has been shown to induce an
enlargement/fusion of the megasomes (De Souza Leao et al., 1995), to inhibit the megasome cysteine proteases (Alfieri et al., 1989), and to give rise to an accumulation of class II molecules within these organelles (De Souza Leao et al., 1995). After this treatment, uninfected macrophages were cultured in parallel and processed in a similar manner. Preparations were examined by confocal microscopy. (A-C) Three-dimensional reconstruction of an uninfected macrophage. (D-F) 0.5 µm optical section of an infected macrophage. fluorescent signals specific to class II (red staining) and H-2M (green staining) molecules are shown in A, D, and B, E, respectively. Merged images showing colocalization of these two molecules, which appears in yellow, are shown in C, F. Class II molecules are present on the plasma membrane (long arrows) and in internal vesicles, which also contain H-2M molecules (short arrows). In the infected macrophage, which is occupied by two PV (a large one and a small one), both class II and H-2M molecules are present on the PV membrane. In the large PV, they are concentrated in the same area corresponding to the sites of parasite attachment (arrowheads). As shown in D-F for the small PV, in very rare cases, PV-associated H-2M molecules are distributed in a polarized fashion whereas class II molecules of the same compartment are apparently evenly distributed (double arrowheads). n, macrophage nucleus. The asterisks indicate the centre of the large PV. The micrographs shown are representative of four similar experiments. Bar, 2 µm.

**Fig. 2.** Double immunofluorescence labelling of H-2M and of MHC class II molecules in uninfected and infected macrophages. Macrophages from BALB/c mice were infected with four amastigotes of *L. amazonensis* per host cell. IFN-γ (25 U/ml) was added after infection as indicated in the legend to Fig. 1. At 48 hours post-infection, macrophages were fixed and stained for class II and H-2M molecules as described in the legend to Fig. 1B. In each experiment, uninfected macrophages were cultured in parallel and processed in a similar manner. Preparations were examined by confocal microscopy. (A-C) Three-dimensional reconstruction of an uninfected macrophage. (D-F) 0.5 µm optical section of an infected macrophage. Fluorescent signals specific to class II (red staining) and H-2M (green staining) molecules are shown in A, D, and B, E, respectively. Merged images showing colocalization of these two molecules, which appears in yellow, are shown in C, F. Class II molecules are present on the plasma membrane (long arrows) and in internal vesicles, which also contain H-2M molecules (short arrows). In the infected macrophage, which is occupied by two PV (a large one and a small one), both class II and H-2M molecules are present on the PV membrane. In the large PV, they are concentrated in the same area corresponding to the sites of parasite attachment (arrowheads). As shown in D-F for the small PV, in very rare cases, PV-associated H-2M molecules are distributed in a polarized fashion whereas class II molecules of the same compartment are apparently evenly distributed (double arrowheads). n, macrophage nucleus. The asterisks indicate the centre of the large PV. The micrographs shown are representative of four similar experiments. Bar, 2 µm.

enlargement/fusion of the megasomes (De Souza Leao et al., 1995), to inhibit the megasome cysteine proteases (Alfieri et al., 1989), and to give rise to an accumulation of class II molecules within these organelles (De Souza Leao et al., 1995). After this treatment, amastigotes with H-2M-containing vesicles were more numerous, the size of the vesicles was apparently enlarged, and the associated specific fluorescence signal was stronger (Fig. 4D-F). As expected, the relatively large H-2M-containing structures were also class II positive. Similar results were obtained with macrophages infected with amastigotes of *L. amazonensis* or *L. mexicana* and then incubated with Z-Phe-AlaCHN₂, or if amastigotes, before infection, were treated for 15 hours with Z-Phe-AlaCHN₂ or Z-Phe-PheCHN₂ and used to infect macrophages previously activated with IFN-γ (data not shown). Taken together, these data are in agreement with the localization of H-2M molecules in megasomes and their degradation by parasite cysteine proteases.

**Effect of the infection with *L. amazonensis* amastigotes on the steady state expression of classical and non-classical MHC molecules and of Ii chains**

Having established that amastigotes of several *Leishmania* species internalized some of the molecules involved in antigen presentation, we wondered whether this could alter the pool of these molecules in infected macrophages. Lysates from uninfected macrophages and from macrophages infected for 48 hours with *L. amazonensis* amastigotes were therefore prepared and analysed by immunoblotting for the relative expression of I-Aα, I-Eα, H-2Mβ, Iip31 and Iip41 chains. Fig. 5 shows that the amount of these different chains was strongly increased after IFN-γ treatment of the macrophages and that infection did not impair the overall I-Aα and I-Eα chain expression, whose levels were comparable in uninfected and infected macrophages or even higher in some experiments (data not shown). However, both Ii and H-2Mβ chains were expressed at a lower level in infected macrophages. If the decrease of the latter could be due to their internalization and degradation by amastigotes, other explanations must account for the lower Ii chain expression, since these chains, under normal conditions, were shown not to be internalized by the parasites (De Souza Leao et al., 1995). Similar findings were obtained for macrophages isolated from BALB/c and C57BL/6 mice (Fig. 5).

**Stability of the MHC class II molecules expressed by infected macrophages**

The results presented above and previous findings (for a review, see Antoine et al., 1998) demonstrate that PV contain all the components required for the formation of stable class II-parasite peptide complexes. To determine whether the closed relationship between the parasites and the molecules of the Ag presentation machinery leads to the formation of a greater amount of stable peptide-class II complexes, infected macrophages were lysed and lysates subjected to SDS
were analysed by protein immunoblotting using anti-I-A

peptide-class II complexes, but not stable complexes (Germain
dissociation of the empty class II molecules and unstable

Fig. 3. H-2M location in PV harbouring various Leishmania species. Macrophages from BALB/c mice were infected with amastigotes of L. mexicana (A), L. major (B) or L. donovani (C), with a multiplicity of four parasites per host cell. IFN-γ (25 U/ml) was added after infection as indicated in the legend to Fig. 1. 48 (B,C) or 72 (A) hours after infection, macrophages were fixed and stained for H-2M using purified anti-H-2Mβ rabbit antibody and a fluorescein conjugate. Nucleic acids were stained with propidium iodide (red staining; large arrowheads, macrophage nuclei; small arrowheads, parasite nuclei and kinetoplasts). Cell preparations were analysed by confocal microscopy. 0.3 μm optical sections are shown. H-2M present on the PV membrane (arrows) are detected solely at the level of amastigote-binding sites (A) or are evenly distributed (B), or else are mainly concentrated at one pole of the compartment (C). The asterisks indicate the centre of the two large PV harbouring L. mexicana (two small PV containing each a single parasite are also present in the section). The micrographs shown are representative of two similar experiments. Bar, 2 μm.

treatment at room temperature before electrophoresis. These conditions have been previously described as causing dissociation of the empty class II molecules and unstable peptide-class II complexes, but not stable complexes (Germain and Hendrix, 1991). After electrophoresis, macrophage lysates were analysed by protein immunoblotting using anti-I-αβ immune serum. As expected (Germain and Hendrix, 1991), the stable αβ dimers were much more abundant in macrophages from C57BL/6 mice than in macrophages from BALB/c mice (Fig. 6). However, in contrast to what has been described for APC loaded with certain exogenous Ag (Germain and Hendrix, 1991), no clear increase of these forms could be detected after infection of macrophages with amastigotes, regardless of the MHC haplotype (Fig. 6), suggesting that, under these in vitro conditions, amastigotes are not an important source of antigenic peptides. Furthermore, no high molecular weight complex involving MHC class II molecules could be detected by this technique, indicating very likely that large parasite molecules do not stably interact with class II molecules of these haplotypes (data not shown).

Distribution of PV-associated MHC class II molecules in H-2M-deficient macrophages infected with L. amazonensis amastigotes

The colocalization of both class II and H-2M molecules at the level of amastigote-binding sites and the internalization of these molecules by amastigotes prompted us to ask whether the polarization of PV-associated class II molecules and their capture by the parasites were dependent upon the presence of H-2M. H-2M molecules (or the human homologue HLA-DM) were recently shown to associate with class II molecules (Sanderson et al., 1996; Schafer et al., 1996; Denzin et al., 1996; Stebbins et al., 1996; Kropshofer et al., 1997a) and to catalyse CLIP release and antigenic peptide binding to class II molecules (for reviews, see Roche, 1995; Kropshofer et al., 1997b). Thus, H-2M molecules could be involved in the fate of PV-associated class II molecules either directly through interactions with parasite components or indirectly by promoting the binding of parasite molecules in the peptide-binding groove of class II molecules. To test these possibilities, macrophages from H-2b mice knockout for the Mα gene (H-2Mat0/0) were prepared and used as Leishmania host cells. The absence of functional H-2M molecules in these mice was demonstrated in previous studies (Miyazaki et al., 1996; Martin et al., 1996; Fung-Leung et al., 1996). However, the presence of H-2Mβ chains could still be detected by immunofluorescence in uninfected as well as infected macrophages of these mice, although in fewer cells than in macrophage cultures from C57BL/6 mice (H-2a). The weak β chain expression in H-2Mat0/0 macrophages was confirmed by western blot analysis (data not shown). Furthermore, PV of infected H-2Mat0/0 macrophages were very rarely stained for H-2Mβ chain and in these cases staining was homogeneously distributed in the PV membrane, rather than being polarized towards the amastigote-binding sites (data not shown).

The fate of PV-associated MHC class II molecules in infected H-2M-deficient macrophages was monitored by confocal microscopy of cell preparations stained with the mAb Y-3P. As controls, H-2M+/+ macrophages from C57BL/6 mice were processed in parallel. In both kinds of macrophages, PV-associated class II molecules exhibited a very similar distribution, namely a preferential localization in the zones of parasite attachment in the PV membrane as well as in intraparasite vesicles (Fig. 7A,D). We concluded that H-2M is not required for the polarization of class II in the PV membrane nor the internalization of these molecules by L. amazonensis amastigotes.

Internalization of I-Aβ-CLIP and of I-Aβ-lip10 complexes by intracellular L. amazonensis amastigotes

In previous studies, splenocytes from H-2M-deficient mice of the H-2b haplotype were shown to be unable to present native antigens to class II-restricted T cell hybridomas and to have a reduced capacity to present antigenic peptides to the same hybridomas, due to the fact that most of their class II molecules are occupied in a stable manner with the peptide CLIP (Miyazaki et al., 1996; Martin et al., 1996), which exhibits a strong affinity for I-Aβ molecules (Sette et al., 1995). In the present study, we took advantage of this characteristic to test whether I-Aβ-CLIP complexes could be detected within PV and internalized by the parasites in spite of the blocked peptide-binding groove of class II. Expression of these complexes by uninfected and infected macrophages derived from the mutant mice or from normal C57BL/6 mice used as positive controls was first studied by immunofluorescence. Cell preparations were stained with the mAb 30-2, which reacts with CLIP-
associated I-A\(^b\). As expected, this antibody did not react with wild-type macrophages (Figs 7B, 8A) because of the short half-life time of the I-A\(^b\)-CLIP complexes under normal conditions. However, it stained about 30% and 60% of uninfected and infected H-2M-deficient macrophages, respectively, on condition that they had been activated by IFN-\(\gamma\) (Fig. 8B). Whereas uninfected macrophages were very weakly stained at the level of their plasma membrane and in intracellular compartments (data not shown), staining associated with infected macrophages was stronger and mainly confined to the PV membrane and internal vesicles of parasites (Fig. 7E). Double immunofluorescence labelling with the mAb 2A3-26, which stains the amastigote plasma membrane, and with the mAb 30-2, clearly showed that the 30-2\(^+\)-vesicles were located within parasites (data not shown). These studies thus revealed that \textit{L. amazonensis} amastigotes can endocytose MHC class II molecules whose binding pocket is occupied by a peptide of non-parasite origin.

To determine whether amastigotes could also internalize class II molecules still complexed with I\(\text{I}\) chain fragments longer than CLIP, macrophages were incubated, after infection, with Z-Phe-AlaCHN\(_2\), which has been shown, like other cysteine protease inhibitors, to partially block the degradation of invariant chains and to induce, in murine cells, the accumulation of class II molecules bound to a 10-12 kDa NH\(_2\)-terminal I\(\text{I}\) chain fragment (lip10; Amigorena et al., 1995; De Souza Leao et al., 1995; Brachet et al., 1997). Furthermore, lip10 fragment has been previously detected within parasites under these conditions (De Souza Leao et al., 1995). The lip10 fragments contain the CLIP region and their complexes with I-A\(^b\) are recognized by the mAb 30-2 (Morkowski et al., 1995). Fig. 8A demonstrates that, under these conditions, a high percentage of infected macrophages from H-2M+/+ C57BL/6 mice were stained with 30-2. A lower percentage of uninfected macrophages from these mice were also stained with 30-2. The difference observed between uninfected and infected macrophages could be due to the fact that in the latter, the 30-2\(^+\) class II molecules were concentrated in the PV membrane and also accumulated in amastigotes (Fig. 7C). H-2M deficient macrophage populations treated with Z-Phe-AlaCHN\(_2\) displayed percentages of 30-2\(^+\) cells very similar to untreated populations (Fig. 8B). However, the staining was stronger, especially in infected macrophages where I-A\(^b\)-lip10 complexes were mainly detected within parasites (Fig. 7F).

![Fig. 4](image_url)

**Fig. 4.** Colocalization of H-2M and of classical class II molecules in internal structures of \textit{L. amazonensis} amastigotes. Macrophages from BALB/c mice were infected with amastigotes of \textit{L. amazonensis} with a multiplicity of four parasites per host cell. IFN-\(\gamma\) (25 U/ml) was added after infection as indicated in the legend to Fig. 1. 28 hours after infection, macrophage cultures were treated with either 0.2% DMSO (controls) or 5 \(\mu\)M Z-Phe-AlaCHN\(_2\) (ZFACHN\(_2\)). 20 hours later, cell preparations were fixed, double stained for class II and H-2M as described in the legend to Fig. 1B and then analysed by confocal microscopy. Optical sections (0.3 \(\mu\)m thickness) of the perinuclear areas of the cells are shown. (A-C) Macrophage not treated with the protease inhibitor. Two PV are present in this section. (D-F) Macrophage incubated with ZFACHN\(_2\). A single PV is present in this section. Class II (red staining) and H-2M (green staining) signals are shown in (A,D) and (B,E), respectively. The images presented in C and F were obtained by merging the corresponding green and red images. In these panels, colocalization of class II and H-2M molecules is seen in white. Both molecules are detected on the PV membrane at the level of parasite attachment (arrows) but also in structures located in the posterior pole of the parasites (arrowheads). These structures are larger and more strongly stained for class II and H-2M molecules in macrophages treated with the cysteine protease inhibitor (D-F). n, macrophage nucleus. The asterisks indicate the centre of the PV. The micrographs are representative of four similar experiments. Bar, 2 \(\mu\)m.
a whole, these results clearly indicate that *L. amazonensis* amastigotes are able to endocytose MHC class II molecules complexed with various invariant chain processing intermediates.

As an additional test for the involvement of parasite cysteine proteases in the degradation of class II-peptide complexes present in PV, macrophages from wild-type C57BL/6 mice or from H-2M-deficient mice were infected with amastigotes previously incubated with 5 μM Z-Phe-AlaCHN₂ for 15 hours (or with 0.2% DMSO for control amastigotes). Before the incubation with macrophages, amastigotes were carefully washed to remove the external drug. By this procedure, only cysteine proteases of the parasites known to be involved in the degradation of host cell class II molecules are blocked. Under these conditions, macrophages from H-2M+/+ mice were not stained with 30-2 (Fig. 9), which strongly suggests that parasite cysteine proteases are not involved in the last degradation steps of Ii chains bound to class II molecules (compare with Fig. 8A). In contrast, most of the infected macrophages derived from H-2M-deficient mice were stained with the mAb 30-2 and similar percentages of stained macrophages were obtained whatever the treatment undergone by the parasites (Fig. 9). The staining was mainly located in the PV membrane and in internal parasite structures. However, the latter were much more strongly stained in amastigotes previously incubated with Z-Phe-AlaCHN₂ (data not shown). Therefore, these findings are consistent with the idea that 1-Aβ-CLIP complexes endocytosed by *L. amazonensis* amastigotes are degraded by parasite cysteine proteases located in megasomes.

**DISCUSSION**

In this work, we demonstrate that PV of mouse macrophages infected with various *Leishmania* species and activated with IFN-γ contain, in addition to classical MHC class II molecules, the non-classical MHC class II molecule H-2M. Both molecules very likely originate, at least partly, from late

**Fig. 5.** Steady state expression of I-A, I-E, Ii and H-2M molecules in uninfected macrophages or macrophages infected with *L. amazonensis* amastigotes. Macrophages from BALB/c or C57BL/6 mice, cultured in 100 mm tissue culture dishes, were infected at a multiplicity of four or five parasites per host cell (I). Macrophage cultures were treated with IFN-γ or control medium, as indicated in the legend to Fig. 1. At 48 hours post-infection, macrophages were recovered by scraping with 0.15 M NaCl containing protease inhibitors or were lysed with Chaps-containing buffer. Laemmli’s sample buffer with 2-mercaptoethanol was then added and samples heated for 5 minutes at 100°C, before electrophoresis on 12% acrylamide gels and immunoblotting with rabbit anti-I-Aα, anti-I-Eα or anti-H-2Mβ immune serum, or with the anti-Ii mAb In-1 and adequate alkaline phosphatase conjugates. Uninfected macrophages were run in parallel and processed in a similar manner (U). Lanes were loaded with 5.4×10⁵ (BALB/c) or 2×10⁵ (C57BL/6) lysed macrophages. Positions of molecular mass markers (kDa) are indicated on the right of the panels. The data are representative of six experiments.

**Fig. 6.** Detection of SDS-stable class II αβ dimers in lysates of uninfected macrophages or macrophages infected with *L. amazonensis* amastigotes. Macrophages from BALB/c or C57BL/6 mice, cultured in 100 mm tissue culture dishes, were infected with four amastigotes per host cell (I) and then treated with IFN-γ as indicated in the legend to Fig. 1. At 48 hours post-infection, macrophages were lysed with Chaps-containing buffer (BALB/c) or NP-40-containing buffer (C57BL/6), after which Laemmli’s sample buffer was added. Samples were left for 20-30 minutes at room temperature before electrophoresis (not boiled, NB) or heated immediately for 5 minutes at 100°C (boiled, B). They were then electrophoresed on 10% (BALB/c) or 12% (C57BL/6) acrylamide gels and electrotransferred to Immobilon-P (BALB/c) or Hybond-C Extra (C57BL/6) membrane. Blots were incubated with rabbit anti-I-Aα immune serum and an adequate alkaline phosphatase conjugate. Lysates of uninfected macrophages were electrophoresed in parallel and processed in a similar manner (U). Lanes were loaded with 1.2×10⁶ (BALB/c) or 2×10⁵ (C57BL/6) lysed macrophages. Positions of molecular mass markers (kDa) are indicated on the right of the panels and those of monomeric α chains (α) and of SDS-stable αβ dimers (αβ) on the left of the panels. The data are representative of 4-6 experiments.
endosomes and lysosomes which, in APC, were reported to contain a large part of the intracellular class II molecules and almost all H-2M molecules (Kleijmeer et al., 1997), and have been shown to fuse with nascent PV (for a review, see Antoine et al., 1998). This assumption is supported by the fact that we observed in infected macrophages a depletion of H-2M-containing compartments other than PV, notably in macrophages harbouring L. amazonensis, L. mexicana or L. donovani. Otherwise, newly synthesized MHC molecules could also be directly delivered to PV, especially in macrophages exhibiting a depletion of unmodified late endocytic compartments.

Late endocytic compartments of APC, which are enriched in MHC class II and H-2M molecules and which contain the bulk of intracellular stable class II-peptide complexes, are generally thought to be the primary sites for peptide loading (for a review see Geuze, 1998). PV of Leishmania-infected macrophages, which share many characteristics with these late endocytic compartments, can thus be considered as potential sites for antigen processing and peptide binding to MHC class II molecules. In this respect, complexes between a self peptide and I-Aβ molecules specifically recognized by the mAb Y-Ae (Murphy et al., 1992) were detected in PV of L. amazonensis-infected B10.A (5R) macrophages (Antoine et al., 1998; J.-C. Antoine, unpublished results). However, the presence in PV of complexes between class II and parasite peptides still remains to be demonstrated. In any case, if the formation of such complexes occurs in Leishmania-infected macrophages, this does not result in a clear increase in the amount of class II molecule compact conformers.

Paradoxically, our finding that, in PV harbouring L. amazonensis or L. mexicana, both class II and H-2M molecules are very often clustered at the level of amastigote-binding sites suggested instead that these molecules are linked and, by
inference, that a large proportion of PV-associated class II molecules are empty. Indeed, recent studies have shown that HLA-DM (the human homologue of H-2M) has a much higher affinity for empty class II molecules than for, in decreasing order, class II-CLIP complexes, class II-associated with lip10 and class II-peptide complexes (Denzin et al., 1996; Kropshofer et al., 1997a). Alternatively, the clustering of both PV-associated class II and H-2M molecules could reflect, instead of a direct physical interaction, the retention at the level of parasite-binding sites of large multimolecular structures like those described recently in late endocytic compartments of human B lymphocytes and involving HLA-DR, HLA-DM, HLA-DO and several tetraspanin molecules (Hammond et al., 1998). In these large complexes, class II and H-2M are not necessarily directly bound and the state of class II has not yet been determined. More work is therefore required to precisely define the state of PV-associated class II molecules, including those clustered with H-2M. Nonetheless, they appear to be completely devoid of any li chain fragments including CLIP, since they are recognized neither by the mAb In-1, which is specific for the cytosolic tail of invariant chains (Lang et al., 1994a,b) nor, in H-2b macrophages, by the mAb 30-2, specific for CLIP-I-A\textsuperscript{b} complexes.

The mechanism underlying the clustering of class II molecules in the large communal PV remains to be precisely defined, but our present data indicate that it is H-2M-independent, since the phenomenon was still observed in H-2M-deficient macrophages. Furthermore, I-A\textsuperscript{b}-CLIP complexes detected in the PV membrane of H-2M-deficient macrophages of the H-2\textsuperscript{b} haplotype also displayed a restricted distribution at the level of amastigote-binding sites, indicating that this event does not necessarily require interactions between the peptide-binding groove of class II and parasite components. Together, the observations suggest that, if the clustering of PV-associated class II is dependent upon specific interactions they establish with parasite plasma membrane molecules, then the contacts between the two kinds of molecules must occur outside of the class II peptide-binding cleft. Alternatively, if MHC class II and H-2M molecules present in the PV membrane are parts of large multimolecular rafts including other proteins and notably tetraspanins, then it is quite possible that their preferential association with parasite-binding sites is due to interactions between parasite molecules and non-MHC molecules of the rafts. We are currently testing this possibility in our laboratory.

The results presented here also provide evidence that H-2M molecules, like class II molecules, are internalized by amastigotes of L. amazonensis and of L. mexicana and accumulate in megasomes where they are probably degraded

Fig. 8. Expression of I-A\textsuperscript{b}-CLIP complexes or of I-A\textsuperscript{b}-Iip10 complexes by uninfected and infected macrophages derived from either wild-type C57BL/6 mice (A, +++) or C57BL/6x129/Sv mice lacking functional H-2M molecules (B, H-2M+/-). Macrophages were infected with 4 L. amazonensis amastigotes per host cell and then treated with IFN-\(\gamma\) (10 U/ml) or control medium. 28 hours after adding parasites, some cultures were treated with 5 \(\mu\)M Z-Phe-AlaCHN\(_2\) (ZFACHN\(_2\)). The cells were fixed at 48 hours post-infection and stained with the mAb 30-2. Uninfected macrophages were similarly processed and then fixed for immunofluorescence analysis. At least 500 macrophages per cell preparation were counted. Results are the means of two separate experiments and are expressed as the percentage of 30-2 positive macrophages.

Fig. 9. Expression of I-A\textsuperscript{b}-CLIP complexes by H-2M+/- and by H-2M-deficient macrophages infected with amastigotes treated with Z-Phe-AlaCHN\(_2\) (ZFACHN\(_2\)) or DMSO. 24 hours before infection, macrophages were activated with IFN-\(\gamma\) (10 U/ml) or left in control medium. Macrophages were infected with 5 L. amazonensis amastigotes per host cell. Parasites had been previously incubated for 15 hours at 34°C with 0.2% DMSO or with 5 \(\mu\)M ZFACHN\(_2\). 24 hours later, macrophages were fixed and stained with the mAb 30-2 and an appropriate fluorochrome conjugate. Uninfected macrophages incubated with IFN-\(\gamma\) or control medium were cultured in parallel and similarly processed. Cell populations were analysed by immunofluorescence microscopy and at least 500 macrophages per preparation were counted.
by parasite cysteine proteases. Whether H-2M internalization requires the presence of class II molecules is not yet known and the present data are compatible with several possibilities. H-2M could be internalized independently of class II molecules, as parts of complexes with class II molecules, or as parts of larger multimolecular complexes. In contrast, experiments performed with macrophages from H-2M-deficient mice of the H-2b haplotype clearly demonstrated that internalization of classical class II molecules by the parasites is H-2M independent and that the process occurs even if the peptide-binding groove of class II is occupied by non-parasite peptides like CLIP. The internalization process of class II also extends to complexes with lip10 fragments, which accumulate in macrophages treated with Z-Phe-AlaCHN\(_2\) (De Souza Leao et al., 1995) and which are organized in high molecular weight nonamers (I-A-lip10)\(_3\) (Amigorena et al., 1995). Thus, although we have not elucidated the mechanism whereby amastigotes capture class II molecules, it is clear that, like the clustering of PV membrane-associated class II molecules, it does not rest on prior interactions between peptide-binding clefts of class II and parasite molecules.

The biological significance of the internalization of MHC molecules by amastigotes of *L. amazonensis* and *L. mexicana* is unclear. For instance, whether this phenomenon is an adaptive strategy selected by the parasites to subvert the Ag presentation process remains to be shown. In any case, the internalization and subsequent degradation of class II molecules by amastigotes does not result in reduced class II expression by inflected macrophages, which generally contain a greater amount of class II than uninfected macrophages (the present data, and J.-C. Antoine, unpublished results). Higher expression of these molecules after infection could compensate for the loss due to their internalization by amastigotes. In contrast, H-2M molecule expression is reduced after infection. Recent studies suggest that a decreased level of H-2M could affect the formation of peptide-class II complexes (Ramachandra et al., 1996). Thus, according to these data, *Leishmania*-infected macrophages could be affected in their ability to present Ag, at least in part because of their lower content of H-2M. This proposal is not supported, however, by the moderate reduction that we observe and by in vitro experiments indicating that HLA-DM/H-2M acts catalytically rather than stoichiometrically to remove the peptide CLIP from the class II molecules and to load class II with peptides (Sloan et al., 1995; Denzin and Cresswell, 1995; Sherman et al., 1995). What is also important to point out is that the clustering of PV-associated MHC class II molecules and their internalization by amastigotes are clearly observed for *L. amazonensis* and *L. mexicana* but for *L. major* and *L. donovani*, these events either do not occur or occur moderately. Yet, *L. major* and *L. donovani*, like *L. amazonensis* and *L. mexicana*, also efficiently downregulate MHC class II presentation of exogenous Ag (Prina et al., 1993; Fruth et al., 1993; Lytton et al., 1993) and of parasite Ag (Wolfram et al., 1995, 1996; Prina et al., 1996; Kima et al., 1996; McMahon-Pratt et al., 1998; Courret et al., 1999). So, either these phenomena have nothing to do with the downregulation of class II presentation, or *Leishmania* species have evolved different strategies to thwart the Ag presentation process.

In summary, the further characterization of *Leishmania*-infected macrophages provides evidence that H-2M, a non-classical MHC class II molecule, is a component of the PV membrane just like the classical MHC class II molecules, in macrophages stimulated with IFN-γ. It also indicates that both non-classical and classical class II molecules are internalized by amastigotes of some *Leishmania* species via a process that does not bring into play the peptide-binding groove of the latter. It must also be stressed that the various *Leishmania* species examined seem to interact differently with PV-associated MHC molecules. Indeed, the clustering of MHC molecules at adhesion foci, as well as the internalization of these molecules by amastigotes, vary considerably from a species to another. Whether these differences are related to the kinds of immune responses and to the clinical signs induced by the various *Leishmania* species is at present unknown but should be quite interesting to explore.

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