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Class II MHC/Peptide Interaction in Leishmania donovani Infection: Implications in Vaccine Design

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We show that Leishmania donovani–infected macrophages (MΦs) are capable of stimulating MHC class II (MHC-II)–restricted T cells at 6 h of infection. At 48 h, infected MΦs (I-MΦs) failed to stimulate MHC-II–restricted T cells but not MHC class I–restricted ones, in contrast to normal MΦs. Such I-MΦs could stimulate T cells at a higher Ag concentration, indicating that general Ag processing and trafficking of peptide–MHC-II complexes are not defective. Analysis of the kinetic parameters, like “k_on” and “k_off” showed that peptide–MHC-II complex formation is compromised in I-MΦs compared with normal MΦs. This indicates interference in loading of the cognate peptide to MHC-II, which may be due to the presence of a noncognate molecule. This notion received support from the finding that exposure of I-MΦs to low pH or treatment with 2-(1-adamantyl)-ethanol, a molecule that favors peptide exchange, led to T cell activation. When treated with 2-(1-adamantyl)-ethanol, splenocytes from 8 wk–infected BALB/c mice showed significantly higher antileishmanial T cell expansion in vitro compared with untreated controls. Hence, it is tempting to speculate that high, but not low, concentrations of cognate peptide may favor peptide exchange in I-MΦs, leading to expansion of the antileishmanial T cell repertoire. The results suggest that a high Ag dose may overcome compromised T cell responses in visceral leishmaniasis, and this has an important implication in therapeutic vaccine design.

Visceral leishmaniasis or kala-azar is a complex disease caused by the intracellular protozoan parasite Leishmania donovani. The antileishmanial immune repertoire is dependent on a variety of factors, such as progression of infection (1), genetic background (2), and interplay of host-protective versus disease-promoting cytokines (3). The L. donovani parasites replicate within macrophages (MΦs) and presumably within the dendritic cells (DCs) in the disease model (3). During their intracellular life cycle, the parasites cause a variety of defects in cellular homeostasis (4). Previously, we showed that L. donovani–infected BALB/c mice display, at early stages, a strong antileishmanial delayed-type hypersensitivity (DTH) response that wanes with time. When purview T cells derived from the DTH-negative phase are transferred with leishmanial Ag to normal mice, a DTH response is observed (1), suggesting a possible defect at the level of APCs. Further, Leishmania homolog of receptors for activated C-kinase (LACK)/I-A^d complexes were detected on the cell surface of Leishmania major–infected DCs under relatively low parasitized conditions but not at high parasitized conditions (5). It is quite intriguing that the peptide–MHC class II (MHC-II) complex is absent under heavily parasitized conditions; this may have bearing on the antileishmanial T cell immune repertoire.

The importance of CD4^+ and CD8^+ T cells in antileishmanial immune response and protection is well studied in L. major (3, 6, 7) and L. donovani systems (8). Both types of T cells produce the host-protective cytokine IFN-γ (9). It is interesting to note that PBMCs of active and cured kala-azar patients produced IFN-γ upon stimulation with leishmanial Ag, but active kala-azar patients simultaneously produced IL-10 (10). IL-10 is known to interfere with TCR-induced IFN-γ production from freshly isolated memory T cells in the absence of APCs (11) and to inhibit generation of IFN-γ– or TNF-α–induced superoxide and NO production (12). IFN-γ binds to the IFN-γR subunits R1 and R2 to transduce the signal to generate reactive species (13). Previously, we showed that the assembly of IFN-γR subunits R1 and R2 is disturbed under parasitized conditions; IFN-γ binds to the receptor but is unable to transduce the signal to kill intracellular L. donovani parasites (14). It was reported that the numbers of CD4^+ T cells decreased, whereas CD8^+ T cells increased, in the PBMCs in acute visceral leishmaniasis (15). It was shown that L. donovani–infected granuloma-derived Kupffer cells present endogenous leishmanial Ag to CD8^+ T cells (16). Mice deficient in MHC class I (MHC-I) presentation are resistant to L. major infection (17), but those deficient in MHC-II are susceptible (18). MHC-II^−/− mice are unable to restrict the growth of L. major, despite having functional CD8^+ T cells (18). Thus, an MHC-II–restricted immune response is most likely essential to control infection. The importance of CD4^+ T cells in helping the expansion of CD8^+ T cells has been well studied in viral infection in mice in whom CD8^+ T cell expansion may be CD4^+ T cell dependent (19–22) or independent (23).

MΦs infected with Leishmania amazonensis display normal levels of MHC-II, but Ag presentation may be inhibited by inefficient loading of peptide to MHC-II (24). Listeria monocytogenes–infected MΦs displayed defective T cell stimulation because of reduced peptide–MHC (p-MHC) stability (25). The reduced stability of HIV-derived p-MHC complexes may explain the mechanism of immune escape in HIV (26). The binding of a second peptide near the peptide-binding groove allosterically modifies the
peptide-binding groove and, thus, may reduce the stability of the peptide–MHC class II (p-MHC-II) complex (27). Several studies demonstrated that the biological effect of a p-MHC–TCR interaction often correlates with affinity and half-life of the p-MHC-II complex, suggesting that the duration of the p-MHC interaction is an important determinant of T cell stimulation (28–30). This study was performed primarily with MΦs because these cells are targets of L. donovani and also act as a prototype of APCs. To our knowledge, we show for the first time that L. donovani–infected MΦs show poor peptide exchange, leading to reduced p-MHC-II formation and stability, which may have a significant bearing on the antileishmanial immune repertoire.

Materials and Methods

Ethics statement

Use of mice was approved by the Institutional Animal Ethics Committee of the Indian Institute of Chemical Biology. All animal experiments were performed according to the National Regulatory Guidelines issued by the Committee for the Purpose of Supervision of Experiments on Animals, Ministry of Environment and Forest, Government of India. The protocol number is SDR/SYR/2007.

Reagents

FBS, penicillin-streptomycin, sodium bicarbonate, HEPES, 2-ME, 1,6-diphenyl-1,3,5-hexatriene, starch, cholesterol, RPMI 1640, Medium 199, Triton X-100, and Giemsa were purchased from Sigma-Aldrich (St. Louis, MO). FITC-conjugated anti-mouse Aβ and an ELISA assay kit for IL-2 assay were purchased from BD Biosciences (San Diego, CA), and Nickel NTA agarose was purchased from Qiagen. All amino acids for peptide synthesis were purchased from Novabiochem (Darmstadt, Germany). The LI sensor chip was purchased from GE Healthcare (Pittsburgh, PA), biotin was purchased from Pierce, and avidin-FITC and anti-avidin-FITC were purchased from Vector Laboratories.

T cell hybridomas

The T cell hybridoma LMR7.5 (Aβ restricted), specific for the 156–173 sequence of LACK (LACK 156–173 peptide), was a gift from Prof. Evelyne Rath (National Institute of Immunology, Delhi, India). The cell line was determined on the basis of positive staining with FITC CD11b, were adherent PECs defined as MΦs. For convenience, adherent PECs are maintained in RPMI 1640 medium supplemented with 10% FCS and 2-ME. The cell line was maintained in RPMI 1640 medium supplemented with 10% FCS and 2-ME (5 × 10^{-5} M) at 37°C with 5% CO₂ in a humidified atmosphere.

mAbs

The Abs used, AMS32.1 (IgG2b κ, reacts with I-A of d, f, g7, i, and v haplotypes) and m24-H cell line specifically recognizing LACK (156–173) complex, were gifts from Prof. Evelyne Moungue. The B3Z CD8+ T cell hybridoma specific for OVA spanning residues 257–274 (SIINFEKL, Kε restricted) was a gift from Dr. Satyajit Rath (National Institute of Immunology, Delhi, India). The cell line was maintained in RPMI 1640 medium supplemented with 10% FCS and 2-ME (5 × 10^{-5} M) at 37°C with 5% CO₂ in a humidified atmosphere.

Animals

BALB/c and C57BL/6 mice were obtained from the animal facility of the institute. The animals were housed under conventional conditions, with food and water ad libitum.

Isolation of peritoneal exudate cells

BALB/c and C57BL/6 (8–10 wk-old) mice were infected i.p. with 3 ml 4% sterile GM-CSF and IL-4 on day 5. After 7 d, the cells were collected, and 2 × 10⁶ cells were transferred into a six-well plate and used as APCs.

Infection of MΦs and DCs with L. donovani

Stationary-phase promastigotes were used for in vitro infection of MΦs. The MΦs were challenged with L. donovani promastigotes (MΦ/parasite ratio = 1:10) and incubated for 6 h at 37°C. Excess parasites were washed off with serum-free medium. The MΦs were incubated further for either 6 or 48 h, and intracellular parasites were enumerated, as described previously (33). Briefly, at end points, the cover slips were washed with PBS, dried, fixed with 100% ethanol, and stained with 10% Giemsa. The intracellular parasites were enumerated using a microscope, and the results are expressed as the percentage of infected MΦs, as well as the number of parasites/100 MΦs. After 48 h, 85–90% of MΦs were infected with 1000–1200 parasites/100 MΦs. About 85–90% of MΦs were infected after 6 h of infection, and the total number was 500–600 parasites/100 MΦs. Approximately 85% of the normal MΦs (N-MΦs) were infected and MΦs were viable.

Pepitide synthesis and characterization

Amino acid residues 156–173 (ICF8PSLHPIVYSVSGWR) of LACK protein (defined as LACK 156–173 Aβ restricted), 73–78 (VVEEPSISAREIYVM) of LEA complex were determined on the basis of positive staining with FITC CD11b, were adherent PECs defined as MΦs. For convenience, adherent PECs are maintained in RPMI 1640 medium supplemented with 10% FCS and 2-ME. The cell line was determined on the basis of positive staining with FITC CD11b, were adherent PECs defined as MΦs. For convenience, adherent PECs are maintained in RPMI 1640 medium supplemented with 10% FCS and 2-ME (5 × 10^{-5} M) at 37°C with 5% CO₂ in a humidified atmosphere.

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Animals

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Isolation of peritoneal exudate cells

BALB/c and C57BL/6 (8–10 wk-old) mice were infected i.p. with 3 ml 4% starch. After 48 h, peritoneal exudate cells (PECs) were isolated and plated on tissue culture petri dishes or glass cover slips in complete RPMI 1640 medium for 48 h at 37°C in a humidified 5% CO₂ incubator. Nonadherent cells were removed by gentle washing with serum-free medium. The PECs, determined on the basis of positive staining with FITC CD11b, were ~95% CD11b⁺ (31). For convenience, adherent PECs are defined as MΦs henceforth.

Generation of bone marrow–derived dendritic cells

Bone marrow–derived DCs of BALB/c mice were generated as described (32). Briefly, a total of 10⁶ nonadherent bone marrow cells/ml, collected from the tibias and femurs of BALB/c mice, was seeded in a six-well plate in the presence of recombinant mouse (rm)GM-CSF (150 U/ml) and rmIL-4 (75 U/ml), cultured for 72 h at 37°C in a humidified 5% CO₂ incubator, and then supplemented with complete medium and cytokines. Subsequently, cultures were fed with rmGM-CSF and rmIL-4 on day 5.

Membrane as surrogate marker of APCs to stimulate T cells

The T cell–stimulating ability of membrane purified from N-MΦs and infected MΦs was studied in the presence of 5 μM OVA323–339. Typically 10 μg/ml MΦ membrane protein was incubated with T cell hybridoma 3D054:8, the resulting IL-2 production in the culture supernatants was assayed by ELISA.

Immobilization of MΦ membranes on the sensor chip

Surface plasmon resonance (SPR) studies were conducted on a Biacore 3000 machine. Membrane was immobilized on the L1 sensor chip for SPR
studies, as described previously (35, 36). The isolated membranes were immobilized on the surface of the L1 chip to achieve the desired response unit. Briefly, the L1 chip was activated with a short injection of 20 mM CHAPS, and immobilization of the membrane was performed. The membranes prepared from N-Mφs and infected Mφs were immobilized separately on the L1 chip up to 2500 response units at a flow rate of 5 μl/min. Loosely bound membrane was dissociated from the sensor chip surface, and a 5-min injection of 0.1 mg/ml BSA (at a flow rate of 10 μl/min) was given to block nonspecific binding sites (35).

Analysis of p-MHC-II stability and affinity on APC membrane using SPR

The running buffer HBSS (pH 7.2) was used for all experiments. After achieving a stable baseline, kinetic experiments were performed with increasing concentrations (1–2000 nM) of the peptides OVA323–339 and OVA Scramble as analytes, flown over the immobilized membrane surface at a rate of 30 μl/min. The sensogram was fitted using Langmuir 1:1 binding isotherm (Biacore, Piscataway, NJ).

Analysis of p-MHC-II formation and stability on intact cells using FACS

Assay of p-MHC-II formation and stability in intact cells was performed by FACS (38). N-Mφs and infected Mφs were pulsed with 15 μM biotinylated OVA323–339 for various lengths of time at 37°C, and the unbound peptide was removed by washing. Cells were stained with avidin-FITC, followed by biotinylated anti-avidin FITC, and then again with avidin-FITC (following the manufacturer’s protocol, Vector Laboratories). This method allowed the amplification of the signal better than directly labeling with avidin-FITC, which generated poor signals. The MFI of 5000 cells was determined by FACS. The observed rate constant (kobs) was calculated using Eq. 1.

\[
\frac{[\text{MF}]}{[\text{MF}_0]} = 1 - e^{-k_{\text{obs}}t}
\]

(1)

where \( [\text{MF}] \) = MFI value after measurement time \( t \), \( [\text{MF}_0] \) = MFI value after reaching equilibrium, \( k_{\text{obs}} \) = observed rate constant, \( k_a \) = association rate constant, \( k_{\text{off}} \) = dissociation rate constant, \( t \) = time of measurement (s), and \( L \) = concentration of peptide used.

N-Mφs and infected Mφs were pulsed with 5 μM biotinylated OVA323–339 for 16 h at 37°C. The cells were then washed, treated with 0.1% sodium azide for 15 min, and incubated with 50 μM OVA323–339 for 1, 3, 6, 9, 15, 25, 35, or 45 h. koff was calculated considering first-order reaction kinetics (Eq. 3). Cells were stained as described earlier. The MFI of the 5000 cells was determined by FACS.

\[
\frac{[\text{MF}]}{[\text{MF}_0]} = e^{-k_{\text{eff}}t}
\]

(3)

where \( [\text{MF}] \) = number of p-MHC peptide complexes at time \( t \), \( [\text{MF}_0] \) = number of p-MHC complexes at time 0; \( k_{\text{eff}} \) = net rate of dissociation; \( t \) = time (h).

The specificity of OVA323–339 peptide binding to Aβ in intact cells was determined by FACS. N-Mφs were incubated with 5 μM biotinylated OVA323–339 peptide for 16 h at 37°C in the presence of 0, 50, or 250 μM unlabeled OVA323–339, and in the presence of 100 μM LACK156–173 or OVA Scramble peptide. The MFI of 5000 cells was determined by FACS. Cell surface binding of OVA323–339 peptide in intact cells was determined by confocal microscopy. N-Mφs were incubated for 16 h at 37°C with 5 μM biotinylated OVA323–339 peptide. Cells were stained as described earlier.

Analysis of p-MHC-II stability on the cell surface using FACS

Mφs (1 × 10^5) were pulsed with 20 μM LACK156–173 peptide for 6 h at 37°C, washed, and fixed with 4% paraformaldehyde. Then the cells were incubated with 50 μM OVA323-339 (Aβ restricted) to prevent rebinding of the dissociated LACK156–173 peptide (39, 40). The cells were incubated with 400 μl m2C4 culture supernatant for 1 h and stained with goat anti-mouse IgG FITC, as described. This mAb binds specifically to LACK156-173 or OVA Scramble but not to OVA323–339, Aβ (5). The MFI of 5000 cells was measured at 0, 2, 4, 8, and 12 h. koff was measured using Eq. 3. This experiment was repeated three times.

Acid treatment of Mφs

N-Mφs and infected Mφs were incubated with citrate buffer (50 mM Na-citrate and 150 mM NaCl [pH 5]) for 90 min, in the presence of either LACK156-173 or OVA323–339 peptide, and then fixed with 4% paraformaldehyde. T cell stimulation was performed with the fixed Mφs in the presence of increasing concentrations of the respective peptide.

Treatment of infected Mφs with 2-(1-adamantyl)-ethanol

The treatment of infected Mφs was carried out as described (41). Briefly, infected Mφs were treated with 2-(1-adamantyl)-ethanol (Aδa; 500 μM) for 4 h in the presence of 5 μM OVA323–339 peptide. Then the cells were washed with PBS and fixed with 4% paraformaldehyde. T cell stimulation was performed with the fixed Mφs in the presence of 5 μM OVA323–339 peptide, as described previously.

T cell–proliferation assay

Spleens were isolated from infected and uninfected BALB/c mice. Single-cell suspensions of splenocytes, prepared after Ficoll density gradient centrifugation, were resuspended in complete RPMI 1640. Cells were plated in triplicate in 96-well plates at a concentration of 10^5 cells/well, with or without 100 μM Aδa, and allowed to proliferate for 72 h at 37°C in a 5% CO2 incubator, with or without soluble Leishmania Ag (SLA; 5, 10, or 100 μg/ml). At 4 h before harvest, cells were incubated with MTT, and MTT assay was performed to measure T cell proliferation, as described previously (31).

Statistical variation and presentation

Each experiment was performed two or three times. The results are expressed as mean ± SD. For the Student t test was performed using GraphPad prism software (p values < 0.05 were considered significant).

Results

Endogenously derived LACK-specific T cell stimulation by infected Mφs

Infected Mφs were used as APCs, and their ability to stimulate MHC-II–restricted LACK–specific T cell hybridoma (LMR7.5) was studied by measuring IL-2 production. Infected Mφs could stimulate LMR7.5 at 6 h, but not at 48 h, postinfection (Fig. 1), because there was compromised T cell–stimulating ability in the 48-h infected Mφs (I-Mφs). The rest of the studies were carried out with I-Mφs.
Cell surface expression of MHC-II

To show that the inability of I-ΜΦs to stimulate T cells was not due to lack of cell surface MHC-II, cells were stained with FITC-labeled anti-I-Αd (AMS32.1). The binding was monitored by FACS, and the results are expressed as MFI. It was observed that the expression of MHC-II on N-ΜΦs and I-ΜΦs was comparable (Fig. 1, inset), indicating that compromised T cell activation by I-ΜΦs was not due to reduced cell surface expression of MHC-II.

L. donovani infection inhibits MHC-II–restricted, but not MHC-I–restricted, Ag presentation

N-ΜΦs and I-ΜΦs were used as APCs, and their abilities to stimulate MHC-II– and MHC-I–restricted T cell hybridomas were studied in the presence of exogenous Ags by measuring IL-2 production. We used one model Ag (OVA) and one leishmanial Ag (LACK) in this investigation. The anti-LACK T cell hybridoma (LMR7.5, Αd restricted) produced IL-2 in response to a low concentration (2.5 μM) of LACK156–173 peptide in association with N-ΜΦs but not with I-ΜΦs (Fig. 2A), whereas I-ΜΦs could stimulate LMR7.5 in the presence of a high concentration (10 μM) of LACK156–173 peptide. Exactly similar results were observed with anti-OVA T cell hybridomas (3DO54.8, Αd restricted) with corresponding sets of either ΜΦs or DCs (Fig. 2B, 2C). In contrast, I-ΜΦs showed enhanced (~10–20%) stimulation of MHC-I–restricted anti-OVA T cell hybridomas (B3Z, Κb restricted, SIINFEKL peptide specific) at a low Ag concentration compared with N-ΜΦs, although at high Ag concentration I-ΜΦs and N-ΜΦs showed similar T cell stimulation (Supplemental Fig. 1A). Thus I-ΜΦs showed compromised MHC-II–restricted T cell activation but not an MHC-I–restricted one.

I-ΜΦs can present MHC-II–restricted protein Ag at high Ag concentration

T cell stimulation (anti-LACK T cell hybridoma [LMR7.5, Αd restricted]) was measured at a high concentration of LACK protein instead of peptide. It was observed that I-ΜΦs showed compromised T cell stimulation at a low protein Ag concentration (5 μM) with N-ΜΦs. Interestingly, at a high concentration (50 μM) of LACK protein, I-ΜΦs can present Ag as efficiently as N-ΜΦs (Fig. 3).

Isolated membrane preparation from ΜΦs as a surrogate of intact APCs

Because metabolic integrity of APCs is not a prerequisite to present processing-independent sequences (42, 43), we sought to determine whether the APCs could be further disrupted with retention of their function. The study showed that isolated membranes from N-ΜΦs induced IL-2 production from an OVA–specific T cell hybridoma (3DO54.8) in the presence of OVA323–339 peptide. As expected, isolated membranes from I-ΜΦs showed ~10-fold reduced T cell–stimulating ability compared with membranes from N-ΜΦs (Supplemental Fig. 2). Thus, the isolated membranes of APCs can act as surrogates for intact APCs.

Analysis of p-MHC-II complex formation on intact cell surface

We studied p-MHC-II complex formation and stability on live cells by FACS using labeled OVA323–339 peptide (40, 44). To show that OVA323–339 peptide binds to the cell surface, live cells were incubated with labeled OVA323–339 Peptide, and the binding was monitored by confocal microscopy. The peptide was observed to bind to the cell surface (Fig. 4A, inset). The association and dissociation of the p-MHC-II complex on the cell surface were monitored by observing the gradual change in MFI using FACS.

The association (kon) and dissociation (k off) rates were determined considering first-order reactions. This revealed that kon and k off values for N-ΜΦs were 25.7 Ms−1 and 10.1 μs−1, respectively. The corresponding values for I-ΜΦs were 1.9 Ms−1 and 35 μs−1 (Fig. 4). The specificity of the peptide binding to live cells was analyzed by competition experiments. There was a gradual de-
crease in MFI upon coincubating the cells with increasing concentrations of unlabeled OVA\textsubscript{323–339} (50 and 250 μM), but there was no change in the MFI in the presence of unrelated peptide lR\textsubscript{73–88} (Ab restricted) (45) or 100 μM OVA\textsubscript{scramble} (Fig. 4B, inset). These observations indicate that the cell surface binding of OVA\textsubscript{323–339} to the membrane-bound A\textsubscript{d} is specific.

Analysis of the kinetics of p-MHC complex formation by SPR

We opted to study p-MHC-II complex formation with a limiting concentration of peptide using SPR. p-MHC-II complex formation was analyzed on the isolated membrane in real time, as described in other systems (35, 36, 46). Increasing concentrations of OVA\textsubscript{323–339} were flown over the immobilized membrane on the L1 chip, and $k_{on}$ and $k_{off}$ were determined using a Langmuir 1:1 binding isotherm. It was observed that $k_{on}$ and $k_{off}$ values were 67.3 M$^{-1}$s$^{-1}$ and 9.32 μs$^{-1}$, respectively, for N-MΦ membrane (Fig. 5A). The corresponding values were 5.6 M$^{-1}$s$^{-1}$ and 32.2 μs$^{-1}$, respectively, for I-MΦ membrane, and 55.9 M$^{-1}$s$^{-1}$ and 10.2 μs$^{-1}$, respectively, for 6-h I-MΦ membrane (Fig. 5B, 5C). To show the specificity of interaction between I-A\textsubscript{d} and OVA\textsubscript{323–339} peptide, similar experiments were performed using OVA\textsubscript{scramble} peptide (scramble OVA\textsubscript{323–339} peptide). The $k_{on}$ and $k_{off}$ values with OVA\textsubscript{scramble
The kon and koff values were 339 M s⁻¹ and 1.29 M s⁻¹, respectively, for I-MΦ membrane (Supplemental Fig. 1C).

Reduced kinetic stability of p-MHC-II complex in L. donovani infection

To authenticate the reduction in p-MHC-II complex formation and stability by I-MΦs, p-MHC-II complex formation was measured by direct demonstration on the cell surface using a LACK156–173-A¹ complex-specific mAb (m2C44). The binding of m2C44 was monitored using an FITC-labeled secondary Ab. The presence of the LACK156–173-A¹ complex was expressed in terms of MFI, and the rate of decrease in MFI on I-MΦs was 1.77 M s⁻¹ and 192 M s⁻¹ (Fig. 5D). Thus, the kon and koff of the complex were ~12-fold slower and ~3.5-fold faster in I-MΦs compared with N-MΦs, and the corresponding values for 6-h I-MΦs were very similar to those of N-MΦs.

The binding of SIINFEKL peptide to MHC-I was measured by SPR. The kon and koff values were 339 M s⁻¹ and 30.1 M s⁻¹, respectively, for N-MΦ membrane (Supplemental Fig. 1B) and 390 M s⁻¹ and 28.9 M s⁻¹, respectively, for I-MΦ membrane (Supplemental Fig. 1C).

Enhancement of T cell stimulation by I-MΦs upon exposure to low pH

At low pH, MHC-II undergoes a conformational change, and the peptide exchange is faster (47); therefore, I-MΦs were treated with a low pH buffer (I-MΦ acid strip). N-MΦs, I-MΦs, and I-MΦ acid strip were used as APCs to stimulate T cells in the presence of increasing concentration of the peptide (0.5, 2.5, and 5 M) LACK156–173 peptide or 0.5, 2, and 5 M OVA323–339 peptide. At low pH (I-MΦ acid strip), the rate of dissociation of p-MHC-II complex was ~11-fold faster on I-MΦs compared with N-MΦs under identical conditions.

We also assessed the availability of the LACK156–173-A¹ complex on the cell surfaces of MΦs on the basis of their ability to stimulate anti-LACK T cell hybridomas (LMR7.5). The T cell–stimulating ability was measured by IL-2 production. At the “0-h” (6 h of pulsing with LACK156–173 peptide) time point, IL-2 production from anti-LACK T cells in the presence of N-MΦs was ∼40% less when driven by I-MΦs compared with N-MΦs. The rate of decrease in IL-2 production in I-MΦs was 17.1 M s⁻¹, whereas it was 1.29 M s⁻¹ for N-MΦs (Fig. 6B).
**I-MΦs show increased T cell stimulation upon treatment with peptide exchange enhancer**

To authenticate that poor peptide exchange may contribute to reduced MHC-II–restricted T cell stimulation, I-MΦs were transiently treated with Ada, and their ability to stimulate T cell hybridomas (3D054.8) was studied in terms of IL-2 production. It is known that Ada enhances loading of peptide to MHC-II (41). The Ada-treated I-MΦs showed ∼7-fold increased T cell stimulation compared with I-MΦs. As a control experiment, N-MΦs were treated with Ada, and the T cell stimulation was comparable to N-MΦs (Fig. 7C).

To authenticate this observation, splenocytes from 8-wk infected mice were treated with Ada in the presence of SLA, and T cell proliferation was measured by IL-2 production upon stimulation with SLA. Ada treatment of splenocytes increased T cell proliferation compared with those without Ada (Fig. 7D). As a control, we treated normal splenocytes with Ada and Con A. Con A treatment enhanced T cell proliferation but Ada treatment failed to enhance T cell proliferation compared with normal in the presence of SLA (Fig. 7E).

**Discussion**

It is evident from our study that MΦs harboring low numbers of intracellular parasites at 6 h postinfection are still capable of stimulating T cells. In contrast, when the intracellular parasite number is higher (at 48 h), the ability of such I-MΦs to stimulate T cells is compromised (Fig. 1). Oddly enough, such I-MΦs are perfectly capable of stimulating MHC-I–restricted T cells (Supplemental Fig. 1A), indicating that the defect is MHC-II specific. MHC-II present on the cell surface of I-MΦs is comparable to that with N-MΦs. In kala-azar there is an expansion of CD8+ T cells but not CD4+ T cells (48). Interestingly, I-MΦs could stimulate MHC-II–restricted T cells at high protein Ag concentration (Fig. 3), which indicated that I-MΦs are not defective in the processing of Ag and trafficking of p-MHC-II complex to the cell surface. This observation is in agreement with those of other investigators (49). We then measured the kinetic parameters of p-MHC-II complex formation and stability in I-MΦs using three independent methodologies and compared these parameters with those in N-MΦs. It is clear from the kinetic studies that the association of immunogenic peptides with MHC-II is slower and dissociation is faster in I-MΦs compared with N-MΦs. This observation is in agreement with the report that fast dissociation of peptide from MHC-II induces T cell anergy (50), as was reported in leishmaniasis (51, 52).

The question remains why there is slow association and fast dissociation of cognate peptide to MHC-II under parasitized conditions. There is a hypothesis that reduced T cell stimulation by I-MΦs, which may be due to MHC-II present in the parasitophorous vacuole, can bind partially unfolded polypeptides expressed at the parasite surface or the parasite lectin or superantigen (53). The binding of noncognate peptide has an allosteric effect on p-MHC-II formation (27), which may enhance dissociation of the cognate peptide (54). The noncognate peptide can bind outside of the peptide-binding groove of MHC-II (27). The presence of a noncognate molecule of higher affinity, if associated with MHC-II, may inhibit peptide exchange and affect the stability of the p-MHC-II. Our study showed that exposure of I-MΦs to low pH enhances MHC-II–restricted Ag presentation in a dose-dependent manner (Fig. 7A, 7B). The adamantane derivative is reported to enhance peptide loading in HLA by transiently stabilizing the peptide-receptive conformation upon binding to Gly at position 86 in the β-chain (41); treatment with Ada enhances the tumor-specific immune response (55). Our study showed that transient treatment of I-MΦs with Ada enhances MHC-II–restricted Ag presentation (Fig. 7C), and treatment of infected splenocytes with Ada enhances SLA-specific T cell responses (Fig. 7D). The Ada-mediated enhancement of T cell responses by I-MΦs could be explained on the basis of a favorable peptide exchange at the MHC-II molecule. Previously, we showed that there is a significant decrease in membrane cholesterol in *L. donovani*–I-MΦs (56), and the transmembrane domain of MHC-II interacts with cholesterol with high affinity (KD 47 nM) (34). Our study also showed that depletion of membrane cholesterol alters conformation of MHC-II that leads to reduced p-MHC-II complex formation (34). It is possible that decreases in membrane cholesterol in *L. donovani* infection alters the conformation of MHC-II, which may contribute to the defective p-MHC-II complex formation when intracellular parasite numbers are high.

In summary, intracellular *L. donovani* parasites reduce MHC-II–restricted Ag presentation as a result of defective peptide exchange ability. Thus, the parasite has evolved a unique mechanism to modify the host CD4+ T cell response, and it is possible to overcome a defective MHC-II–restricted presentation using a high Ag concentration. There is a report that vaccination with a high dose of trivalent inactivated influenza is more effective than a standard dose (57). Thus, in designing an effective therapeutic vaccine against leishmaniasis, for which no vaccine exists, a high dose of Ag may be helpful to overcome the otherwise defective MHC-II–restricted T cell activation.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**

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