Leishmania Antigens Are Presented to CD8$^+$ T Cells by a Transporter Associated with Antigen Processing-Independent Pathway In Vitro and In Vivo

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CD8$^+$ T cells are generated in response to *Leishmania major* (*Lm*) or *Toxoplasma gondii* parasitic infections, indicating that exogenously delivered Ag can be processed for presentation by MHC class I molecules. We show that presentation of *Lm* nucleotidase (NT)-OVA is TAP independent in vivo and in vitro, and is inhibited by chloroquine, but not by proteasome inhibitors. In contrast, the presentation of *T. gondii* P30-OVA relies on the TAP/proteasome pathway. Presentation of OVA- or rNT-OVA-coated beads also bypassed TAP requirement above a certain Ag threshold. TAP was also dispensable for the presentation of wild-type *Lm* Ags to primed CD8$^+$ T cells in vitro. Finally, in vivo priming of CD8$^+$ T cells involved in acquired resistance to *Lm* was not compromised in TAP-deficient mice. Thus, *Leishmania* Ags appear to be confined to an intraphagosomal processing pathway that requires higher concentrations of Ags, suggesting that these parasites may have evolved strategies to impair the efficient endoplasmic reticulum-based, TAP-dependent cross-presentation pathway to avoid or delay CD8$^+$ T cell priming. ***The Journal of Immunology,*** **2006, 177: 3525–3533.**

Presentation of endogenous (self and nonself) Ags in association with MHC class I molecules fulfills essential immunological functions, from thymic depletion of self-reactive T cells, to recognition and destruction of abnormal (tumor) or virus-infected cells. The classical MHC class I processing pathway (reviewed in Refs. 1 and 2) involves the proteolytic digestion of cytosolic Ags by the proteasome and other proteases. Processed peptides are transported to the endoplasmic reticulum (ER)[5] by TAP, a heterodimeric complex composed of TAP1 and TAP2 subunits whose function is ATP dependent. In the ER, β_2-microglobulin/MHC class I and TAP molecules form a complex in the presence of tapasin and other chaperones, and following binding of peptides imported by TAP, stable MHC class I-peptide complexes are exported to the plasma membrane via the constitutive secretory pathway.

Specialized APCs such as macrophages, B cells, and especially dendritic cells (DC) have in addition the capacity to capture, process, and present, in a MHC class I-restricted manner, various exogenous cell-associated Ags, a process referred to as cross-presentation. These exogenous Ags include minor histocompatibility Ags, tumor Ags, or Ags derived from apoptotic, necrotic, and/or virus-infected cells (3–8). Cross-presentation appears designed to insure that CD8$^+$ T cells will encounter viral or tumor Ags on APC that are not themselves directly infected or transformed. Cross-presentation can be extended to a wide range of exogenous Ags derived from pathogens residing transiently or permanently in the phagosome of the host cell, as indicated by the strong CD8$^+$ T cell response induced by infection with *Leishmania*, *Toxoplasma gondii* (*Tg*), *Trypanosoma cruzi*, *Mycobacterium tuberculosis*, *Salmonella typhimurium*, *Brucella abortus*, and *Listeria monocytogenes* (9–15). The cross-presentation of Ags derived from intracellular pathogens might occur as described for other exogenous cell-associated Ags, via the uptake of infected cells by DC, or by direct infection of the DC. In either case, processing of Ags in many cases requires a phagosome-to-cytosol pathway that is a constitutive feature of macrophages and DC (16–19), or else is facilitated by pathogen-mediated lysis of the phagosome, as occurs during infection with *Listeria* or *T. cruzi* (20, 21). Dependence on TAP is taken as evidence that translocation of Ags to the cytosol is required, whereas TAP-independent cross-presentation indicates that peptide ligands are generated and loaded onto MHC class I directly in the phagosome (22) or on the cell surface following peptide regurgitation (12, 23). More recently, we and others (16–18) have described a novel phagosome-associated, ER-based cross-presentation pathway by which OVA, soluble or coated on latex beads, is retrotranslocated to the cytosol and targeted to a phagosome-associated proteasome for degradation. The peptides are then transported by TAP back to the phagosome, and onto the MHC class I/peptide-loading complex.

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[5] Abbreviations used in this paper: ER, endoplasmic reticulum; DC, dendritic cell; DLN, draining lymph node; HFF, human foreskin fibroblast; KO, knockout; *Lm*, *L. major*; LN, lymph node; MOI, multiplicity of infection; NT, nucleotidase; *Tg*, *T. gondii*; WT, wild type.

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As *Leishmania* parasites appear to reside in a similar phagolysosomal compartment as latex beads, we were interested to know whether *Leishmania* Ags can be processed for cross-presentation using the same phagosome-to-cytosol pathway. The manner in which cross-presentation of *Leishmania* Ags is achieved is especially relevant because in clinical studies of cutaneous leishmaniasis, efficient priming of $CD8^+$ T cells and their presence within healing lesions have been a consistent finding (24–26). Furthermore, in experimental infection models of *L. major* (*Lm*), $CD8^+$ T cells are required for the control of primary infection in the skin and in resistance to secondary challenge (13, 27, 28). Finally, primed $CD8^+$ T cells from these mice can be stimulated in vitro to secrete IFN-γ using *Lm*-infected DC (13). In studies designed to monitor the activation and homing of *Leishmania*-specific $CD8^+$ T cells at a clonal level, an *Lm* strain engineered to secrete a chimeric OVA protein (*Lm* nucleotidase (NT)-OVA) was able to induce priming of OVA-specific TCR transgenic $CD8^+$ T cells (OT-I) following infection of DC in vitro and during the course of low dose infection in the skin (29). In the present studies, *Lm* NT-OVA parasites were used to specifically follow the processing of the SIINFEKL peptide and its presentation to OT-I T cells. The participation of TAP was evaluated in vitro and in vivo for the cross-presentation of *Lm* NT-OVA, and further extended to the presentation of wild-type (WT) *Lm* Ags to $CD8^+$ T cells in vivo. Mechanisms for *Lm* Ag cross-presentation by DC are compared with other sources of Ag originating within phagocytic or vacuolar compartments, including OVA-coated beads and OVA-expressing *Tg* parasites.

Materials and Methods

Mice

C57BL/6 mice were purchased from the Division of Cancer Treatment, National Cancer Institute. B6.SJL congenic mice, TAP1 knockout (TAP KO) (backcrossed for at least five generations to C57BL/6) mice, and RAG1-deficient OT-I $CD8^+$ TCR transgenic mice were purchased from Taconic Farms. All mice were maintained in a National Institute of Allergy and Infectious Diseases animal care facility under specific pathogen-free conditions and were treated in accordance with the regulations and guidelines of the Animal Care and Use Committee of the National Institutes of Health.

Parasites, intradermal inoculation, and estimation of parasite load

Lm clone V1 (MHOM/IL/80/Friedlin) and *Lm* NT-OVA transgenic parasites were grown, as previously described (29). Infective-stage promastigotes (metacyclics) were isolated from stationary cultures (4- to 5-day old) by density centrifugation on a Ficoll gradient (30). Metacyclic promastigotes (10^4) were inoculated intradermally into the ear dermis using a 27.5-gauge needle in a volume of ~5 μl. The evolution of the lesion was monitored by measuring the diameter of the induration of the ear lesion with a direct-reading vernier caliper (Thomas Scientific). Parasite titrations were performed on ear tissue homogenates obtained, as previously described (13). The number of viable parasites in each sample was determined from the highest dilution at which promastigotes could be grown out after 7 days of incubation at 26°C.

The RH strain of *Tg* was maintained by serial passage on primary human foreskin fibroblasts (HFF) cultured at 37°C in DMEM (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FBS. Parasites were harvested after 3–5 days in culture when the host cell layer reached ~80% lysis. Parasites were purified from HFF cell debris by centrifugation at 50 × *g* for 10 min, followed by 10 min at 1600 × *g*, and washed with PBS.

OVA-expressing Tg *parasites*

Plasmid construct. The *ptubP30-OVA/SagCAT* plasmid is derived from the *ptubP30-GFP/SagCAT* previously described by Striepen et al. (31). This construct contains the major surface Ag (P30, Sag-1) of *Tg*, truncated to remove the GPI anchor domain, thus allowing secretion of the recombinant protein. The OVA fragment was amplified by PCR from an OVA-containing plasmid template (provided by R. Germain, National Institutes of Health, Bethesda, MD) using the forward 5′-ATC GAC CTA GGG ATC AAG CCA GAG AGC TCA TC-3′ and reverse 5′-AAA ACT GCA GTT AAG GGG AAA CAC ATC TGC C-3′ primers. The PCR product was digested with *Avr*II and *Pst*I and cloned in place of GFP in the *pCAT-GFP* vector. The *Avr*II-*Not*II insert was subsequently cloned into the *ptubP30-GFP/SagCAT* vector. Correct insertion of the fragment was controlled using restriction analysis and DNA sequencing. *Tg* tachyzoites were transfected with the *ptubP30-OVA/SagCAT* plasmid, as described previously (31).

SDS-PAGE and Western blotting. Total parasite lysates were obtained from 5 × 10^6 tachyzoites and lysed in SDS-PAGE sample buffer. For analysis of proteins released by *Tg* tachyzoites, 10^8 cells/ml were cultured overnight in DMEM. Parasites were removed with a 10-min centrifugation at 1,600 × *g*, and 1 vol of supernatants was added to 1 vol of 2× SDS-PAGE sample buffer. Total parasite cell lysates and culture supernatants were analyzed by SDS-PAGE and Western blotting using a rabbit anti-OVA Ab (1/10,000; Sigma-Aldrich), followed with anti-rabbit Ig-HRP (1:3,000; Amersham).

Immunofluorescence. For immunolabeling of *Tg*-derived OVA, parasite-infected HFF cell monolayers grown on glass chamber slides were fixed with methanol. Immunostaining was performed using rabbit anti-OVA polyclonal Ab (Sigma-Aldrich) diluted 1/2000 in 1% (v/v) FBS in PBS, followed with AlexaFluor 488-conjugated anti-rabbit Igs (Molecular Probes) diluted 1/100 in the same dilution buffer.

rNT-OVA protein expression in Escherichia coli

The rNT-OVA protein was expressed and purified as a histidine-tagged protein in *E. coli*. Briefly, the NT-OVA nucleotide sequence was amplified by PCR using the *pKS NEO NT::OVA* plasmid (29) as template and the forward 5′-ATG TGG TGG AGC AAG GGC CAC ATG-3′ and reverse 5′-CTC CAT CTT CAT GCG AGG TAA GTA CAC TTT GAT C-3′ primers. The PCR fragment was cloned into the *pCRT7/CT-TOPO* vector (Invitrogen Life Technologies). The resulting plasmid encodes the NT-OVA protein containing a C-terminal $(His)_6$ tag (rNT-OVA). BL21(DE3) *E. coli* host cells (Novagen) were transformed with this plasmid. rNT-OVA protein was purified by affinity chromatography using Ni-NTA agarose (Qiagen), under denaturing conditions, according to the manufacturer's protocol. Purified rNT-OVA protein was subsequently dialyzed against PBS, concentrated using Centrion-10 concentrator (Millipore), and stored at −20°C. Protein concentration was determined by bicinchoninic acid assay (Pierce) and diluted to 1 mg/ml for adsorption on latex beads.

DC culture and infection

Mouse bone marrow-derived DC were cultured, as described (29), and incubated for 16 h with *Leishmania* metacyclic promastigotes, opsonized by incubation for 30 min at 37°C in 5% fresh normal mouse serum, or irradiated (15,000 rad) *Toxoplasma* tachyzoites (2–4 parasites per DC), or with 0.04–1 nM SIINFEKL peptide as positive control, and further used as APCs cocultured with purified $CD8^+$ T cells from RAG1-KO TCR transgenic OT-I mice. Aliquots of cells were prepared in a cytospin and stained using Diff Quick (Dade Behring) to evaluate the level of infection. For experiments using Ag-coated latex beads (3 μm; Polysciences), typically 10^7 beads/condition were incubated for 2 h at room temperature in different concentrations of Ag, using a 1 mg/ml stock of OVA or rNT-OVA serially diluted in PBS containing 1 mg/ml BSA. Protein-coated beads were added to DC at a 10:1 ratio for 6 h before adding the OT-I $CD8^+$ T cells.

Purification of OT-I $CD8^+$ T lymphocytes, CFSE labeling, and in vitro and in vivo activation

$CD8^+$ T lymphocytes were negatively selected by magnetic separation (MACS system; Miltenyi Biotec), according to the manufacturer's indications. The purity of $CD8^+$ T lymphocytes was >95%. Purified $CD8^+$ T cells were incubated at 2.5–5 × 10^7 cells/ml in PBS with 0.5 μM CFSE (Molecular Probes) for 10 min at 37°C; the reaction was stopped with 10% normal mouse serum; and the cells were washed twice with cold PBS/0.1% BSA. CFSE-labeled $CD8^+$ T cells were plated at 10^6 cells/well in 24-well plates (Costar Life Science Products; Corning Glass) in RPMI 1640/10% FCS, and 3 × 10^5 uninfected, infected, or Ag-pulsed DC were added for 72 h, at which time the cells were fixed in 4% paraformaldehyde.

B6.SJL congenic mice or TAP-1 KO mice received i.v. 2–5 × 10^6 CFSE-labeled purified $CD8^+$ OT-I T cells. The mice were challenged the same day in the pinea of the ear with 10^5 metacyclic promastigotes or 5 μg of SIINFEKL peptide. Five days later, the draining lymph nodes (DLNs) were removed and analyzed by flow cytometry.

Immunolabeling and flow cytometry

Abs used were from BD Pharmingen. Before staining, all of the cells were incubated with an anti-FcRIII/II mAb in PBS containing 0.1% BSA. T cell proliferation was measured by flow cytometry at the single-cell level, as expressed by the intensity of CFSE fluorescence. OT-I $CD8^+$ T cells were identified by characteristic size and granularity, in combination with anti-CD45.2-PerCP, H2-K^b-OVA SIINFEKL tetramer-PE (iTAg, Immunomic; Beckman Coulter), TCR β-chain (PE or CyChrome conjugated), and anti-CD8 (CyChrome or allophycocyanin conjugated) surface staining.

For each sample, between 20,000 and 400,000 cells were analyzed using CellQuest software and a FACSCalibur flow cytometer (BD Biosciences).

ELISPOT

DC were incubated for 1 h with serum-opsonized *Lm* NT-OVA metacyclic promastigotes (10 parasites per DC), or alternatively with *Tg* P30-OVA, or 5 pg/ml SIINFEKL peptide as control, washed, and treated with 150 μM leupeptin, 15 μM pepstatin A, 0.4 μM epoxomicin, 10 μM lactacystin (all from Calbiochem and EMD Biosciences), and 35 μM chloroquine (Fluka) for an additional 10 h at 37°C. After treatment with the inhibitors, DC were washed and used to stimulate primed OT-I T cells obtained at day 10 poststimulation in vitro with SIINFEKL peptide plus 30 U/ml IL-2 (PeproTech).

To evaluate OVA-specific cross-presentation, IFN-γ secretion by primed OT-I T cells was monitored by ELISPOT. $CD8^+$ T cell ELISPOT assays were conducted using 96-well nitrocellulose plates (polyvinylidene difluoride-based membrane plates, ELIIP10SSP; Millipore) precoated with 100 μl/well of 10 μg/ml mouse anti-mouse IFN-γ mAb clone AN18 in PBS (Mabtech) overnight at 4°C. Plates were washed four times and blocked with 10% FBS for 2 h at 37°C. Primed OT-I T cells (5×10^3) and infected DC (1×10^5) were then added in DMEM containing IL-2 (30 U/ml). Cytokine production was detected after 6 h using biotinylated Ab against mouse IFN-γ (clone R4-6A2; Mabtech), followed by alkaline phosphatase-labeled streptavidin (Mabtech). Spots were developed after the addition of alkaline phosphatase substrate and counted using an Immunospot Series 3A Analyzer (Cellular Technology).

$CD8^+$ cell depletion

Mice were inoculated i.p. with 1 mg of rat mAb anti-CD8α (clone 2.43; Harlan Bioproducts for Science) or rat isotype control (clone GL113; Harlan), 1 day prior to challenge and weekly thereafter until termination of the experiments.

Analysis of intradermal lymphocytes

To characterize leukocytes in the inoculation site, the ears were collected, digested, and processed, as described previously (13). Ear cell homogenates were analyzed by flow cytometry for surface (CD8, TCRβ, CD44) and cytoplasmic (IFN-γ, TNF-α, granzyme B, and CD107a) staining. For cytokine determinations, the cells were incubated at 37°C for 4 h in the presence of PMA (50 ng/ml), ionomycin (500 ng/ml), and brefeldin A (10 μg/ml).

Preparation of Leishmania-*specific $CD8^+$ T cells and in vitro recall response*

C57BL/6 or TAP KO mice were inoculated intradermally with 10^4 metacyclic promastigotes (*Lm* clone V1) in the ear dermis. Six to 8 wk later, when the lesions started to heal, single-cell suspensions were obtained from DLNs and spleen, as described previously (13). Primed $CD8^+$ T lymphocytes from the different tissues were positively selected by magnetic separation, and plated at 1.5×10^5/well in round-bottom 96-well plates in RPMI 1640/10% FBS. Uninfected or *Lm*-infected DC (5×10^4) were added for 72 h, at which time the cell culture supernatants were collected for cytokine determination. In some experiments, 10 μg/ml anti-CD8α mAb (2.43) was added to the wells. IFN-γ in culture supernatants was quantitated by ELISA, following the manufacturer's protocol (eBioscience).

Statistical analysis

Statistical significance between geometrical means of various groups was determined using a two-tailed Student's *t* test for independent samples. All data from parasite numbers were log transformed before statistical tests were applied.

Results

Cross-presentation of Lm *NT-OVA by infected DC is TAP independent in vitro*

We have reported previously that bone marrow-derived DC, but not macrophages infected with *Lm* NT-OVA transgenic parasites, were able to prime $CD8^+$ OT-I T cells to proliferate and produce IFN-γ in vitro (29). To begin to address whether NT-OVA might access the conventional MHC class I processing machinery, the requirement for TAP was investigated. DC derived from C57BL/6 or TAP KO mice were incubated with *Lm* 3′NTs, or NT-OVA, and cultured for an additional 72 h with CFSE-labeled OT-I cells. T cell proliferation was measured as an indicator of activation in response to SIINFEKL processing and presentation by DC (Fig. 1*A*). *Lm* NT-OVA-infected TAP KO DC induced OT-I T cell proliferation as efficiently as WT DC, with 54 and 55%, respectively, of cells showing reduced CFSE levels. OT-I T cell proliferation was Ag specific, as neither WT nor TAP KO DC activated OT-I when infected with *Lm* 3′NTs. Levels of IFN-γ were measured as a second marker for T cell activation (Fig. 1*B*). Elevated IFN-γ responses were observed with *Lm* NT-OVA-infected WT DC or TAP KO DC. Background levels were observed with uninfected DC, or DC infected with *Lm* 3′NTs. It was of interest to compare in parallel studies the cross-presentation of *Leishmania* Ags with Ags derived from another intracellular parasite. It was shown previously that *Tg*-infected APC present parasite antigenic peptides on MHC class I to specific $CD8^+$ T cells (32). Tachyzoites transfected with the *ptubP30-OVA/SagCAT* plasmid (Fig. 1*C*) expressed and secreted the ~55-kDa P30-OVA protein, as detected by immunofluorescence on infected DC, using an anti-OVA Ab (Fig. 1*D*) and immunoblotting of parasite cell lysates and culture supernatants (Fig. 1*E*, *lanes 2* and *5*, respectively). DC derived from C57BL/6 or TAP KO mice were incubated for 16 h with irradiated *Tg* WT or P30-OVA, and cultured for an additional 72 h with CFSE-labeled naive OT-I cells. P30-OVA-infected WT DC induced OT-I T cell to proliferate (Fig. 1*A*), with 77% of the $CD8^+$ T cells showing a reduced CFSE content. In contrast, P30-OVA-infected TAP KO DC failed to induce proliferation above background levels (3–4%) observed with WT *Tg*-infected DC. Similarly, activated OT-I T cells secreted IFN-γ in response to P30-OVA-infected DC, but not in response to P30-OVA-infected TAP KO DC (Fig. 1*B*). The level of OT-I proliferation in response to P30-OVA-infected DC was reduced compared with NT-OVA, possibly due to differences in quantities of Ag made by the two model parasites.

Thus, whereas OVA derived from *Tg* appears to follow a phagosome-to-cytosolic pathway to be processed for MHC class I presentation, OVA derived from *Lm* is not dependent on, and may not even use this pathway, based on the comparability of the OT-I activation by infected WT or TAP KO DC. To control for a possible kinetic difference in *Lm* NT-OVA cross-presentation by WT or TAP KO DC, OT-I proliferation was monitored every 12 h for 3 days (Fig. 2*A*). No differences over time were observed, and in each case the initiation of the proliferative response occurred between 48 and 60 h. Furthermore, TAP KO DC cross-presented *Lm* NT-OVA as efficiently as WT DC at all multiplicities of infection (MOI) (Fig. 2*B*), suggesting that similar pathways are used whether the Ag is limiting or in excess. By contrast, TAP KO DC infected with *Tg* P30-OVA failed to activate OT-I T cells to proliferate (Fig. 2*B*) or release IFN-γ (data not shown) at any MOI, suggesting that the requirement for TAP in the cross-presentation of the SIINFEKL peptide by *Tg* P30-OVA-infected DC cannot be bypassed with even high concentrations of secreted Ag.

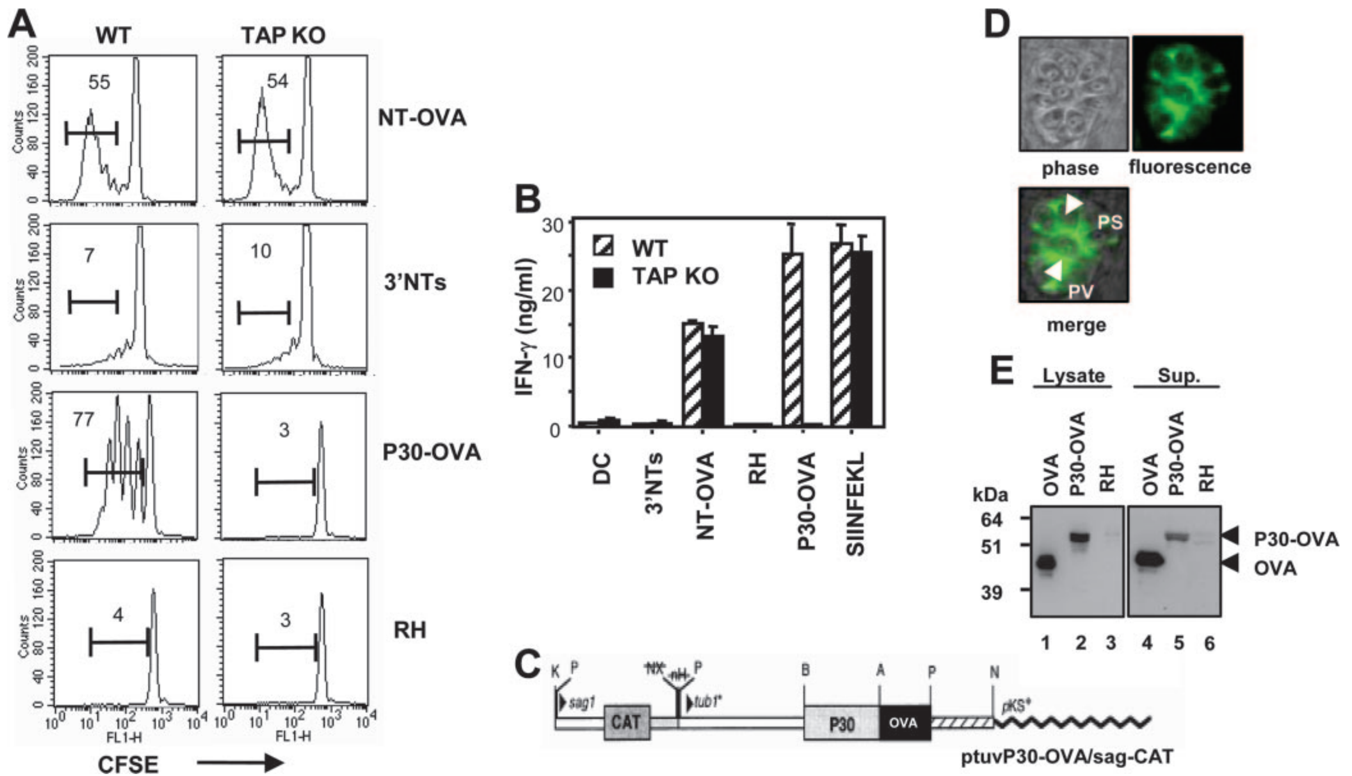

FIGURE 1. TAP dependence for the presentation of *Lm* NT-OVA and *Tg* P30-OVA. CFSE-labeled OT-I cells were incubated with WT or TAP KO DC, uninfected, infected with *Lm* 3′NTs or NT-OVA, *Tg* RH or P30-OVA, or pulsed with 50 pM SIINFEKL peptide. *A*, Intensity of CFSE fluorescence as an indicator of T cell proliferation. Numbers represent the percentage of TCRβ^+CD8$^+$ cells showing a reduced CFSE fluorescence. *B*, IFN-γ levels measured in 72-h culture supernatants. WT DC (▨); TAP KO DC (■). The data shown are the mean ± SD of three replicates, and the experiment is representative of three separate experiments. *C*, *ptubP30-OVA/SagCAT* plasmid. *D*, Expression and secretion of OVA by *Tg* tachyzoites. Immunofluorescence on P30-OVA-infected HFF cell monolayers with a polyclonal rabbit anti-OVA Ab, followed with AlexaFluor488-conjugated anti-rabbit IgG. The arrows indicate: PS, parasite; PV, parasitophorous vacuole. *E*, Western blots showing the reactivity of the anti-OVA Ab with cell lysates or parasite culture supernatants (Sup.) of WT (RH) or P30-OVA-transfected *Tg* tachyzoites, or OVA protein as positive control. The arrows indicate the OVA and P30-OVA fusion proteins.

In contrast, OVA released within model latex bead phagosomes was processed via a TAP-dependent or independent pathway, depending on the concentration of Ag used to coat the beads. At low OVA concentrations, WT, but not TAP KO DC induced OT-I proliferation (Fig. 2*C*). At ~15-fold higher concentration of coated Ag, however, TAP KO DC initiated OT-I activation. Similar numbers of intracellular beads per cell were counted in WT (9.7 ± 1.8) and TAP KO (9.0 ± 0.9) DC. Levels of IFN-γ secreted by activated OT-I T cells paralleled proliferation results (data not shown). These data are consistent with prior studies suggesting a lower efficiency of Ag presentation following uptake of particulate Ags by macrophages or DC lacking TAP (23, 33–35). To address the possibility that the NT-OVA fusion protein secreted by *Lm* could have intrinsic properties allowing it to bypass a requirement for TAP even at low concentrations of released Ag, the cross-presentation of SIINFEKL peptide following uptake of beads coated with rNT-OVA was analyzed (Fig. 2*C*). Similar to OVA, TAP-dependent presentation of rNT-OVA was observed below a certain Ag concentration on the beads, with the TAP KO DC again requiring a greater concentration of Ag (4-fold) to initiate OT-I proliferation. Thus, despite their sequestration within a similar phagolysosomal compartment, the processing of NT-OVA from *Lm* is distinguished from the processing of NT-OVA from latex beads by the absence of a discernable TAP-dependent mechanism. These results suggest that the cross-priming of *Lm*-derived NT-OVA will only proceed when the concentration in phagosomes has reached the threshold required for TAP-independent processing to occur.

Lm *NT-OVA Ag presentation is sensitive to endosomal pH*

Dependence on TAP is taken as evidence that translocation of Ags to the cytosol and processing by the proteasome are required, whereas TAP-independent cross-presentation indicates that peptide ligands are generated by endosomal proteases and loaded onto MHC class I directly in the phagosome. The presentation of *Lm* NT-OVA and *Tg* P30-OVA was evaluated in the presence of a lysosomotropic agent (chloroquine), endosomal protease inhibitors leupeptin (trypsin-like and cystein proteases) and pepstatin A (aspartic proteases), and proteasome inhibitors (lactacystin, epoxomicin). DC were pulsed for 1 h with *Lm* NT-OVA or *Tg* P30-OVA parasites (10 parasites per DC), or with 5 pg/ml SIINFEKL peptide, and further incubated for 10 h in the presence of the inhibitors. The presence of surface MHC I/peptide complexes was evaluated by adding in vitro primed OT-I T cells and counting the number of activated IFN-γ-producing cells 6 h later by ELISPOT. In the representative experiment shown in Fig. 3, the processing of *Lm* NT-OVA by DC was reduced by 60 and 23% in the presence of chloroquine and leupeptin, respectively, while pepstatin A had no inhibitory effect. In three independent experiments, the mean inhibition by chloroquine was 53% ($p < 0.01$). A slight decrease in numbers of OT-I cell-secreting IFN-γ was seen when infected DC were incubated in the presence of proteasome inhibitors epoxomicin (14%) and lactacystin (16%), and these inhibitory effects were not significant when the means of three independent experiments were compared. The SIINFEKL peptide, which does not

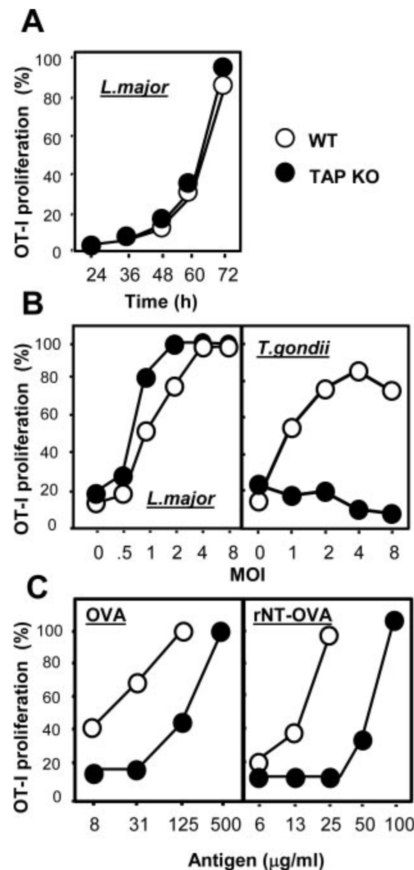

FIGURE 2. Kinetics and dose responses of OT-I cell proliferation in response to different sources of OVA Ag: involvement of TAP. CFSE-labeled OT-I cells were incubated with WT (open symbols) or TAP KO (filled symbols) DC pulsed with *Lm* NT-OVA, *Tg* P30-OVA, and OVA-coated or rNT-OVA-coated latex beads. T cell proliferation was determined as the percentage of OT-I cells showing a reduced level of CFSE fluorescence. *A*, OT-I proliferation followed over time in response to *Lm* NT-OVA. *B*, Proliferation in response to *Lm* NT-OVA or *Tg* P30-OVA at different MOI. *C*, OT-I proliferation in response to OVA- or rNT-OVA-coated beads. The results are representative of two independent experiments.

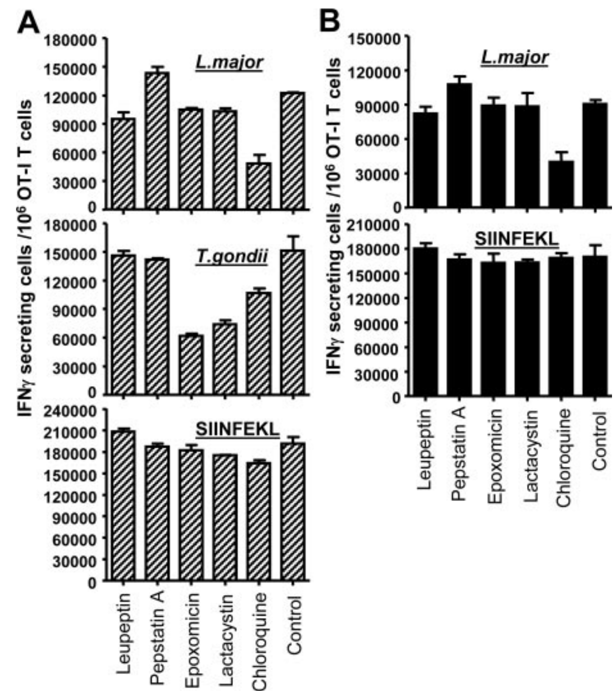

FIGURE 3. Effect of protease inhibitors on *Lm* NT-OVA and *Tg* P30-OVA presentation to OT-I cells. DC (*A*) or TAP KO DC (*B*) were pulsed with *Lm* NT-OVA, *Tg* P30-OVA, or SIINFEKL peptide (5 pg/ml), and further incubated for 10 h in the presence of leupeptin (150 μM), pepstatin A (15 μM), epoxomicin (0.4 μM), lactacystin (10 μM), and chloroquine (35 μM). In vitro-primed OT-I cells were then added to the culture for 6 h, and the number of activated IFN-γ-producing OT-I cells was counted by ELISPOT. The results are representative of three independent experiments.

require processing for presentation on MHC class I molecules, was used to monitor possible side effects of the inhibitors. Leupeptin, pepstatin A, and epoxomicin induced no to low levels of inhibition (0, 2, and 5%), while lactacystin and chloroquine had moderate effects on peptide presentation with 9 and 15% inhibition, respectively. Finally, in contrast to *Lm* NT-OVA, and correlating with the requirement for TAP, the processing and presentation of *Tg* P30-OVA by DC were strongly inhibited by both proteasome inhibitors epoxomicin and lactacystin, with a mean inhibition in three experiments of 53 and 50%, respectively ($p < 0.005$). Leupeptin and pepstatin A had no effect above background (3 and 4%, respectively), while chloroquine moderately inhibited Ag processing (24%; $p < 0.02$), possibly by affecting parasite metabolism and/or survival inside DC.

Lm *NT-OVA-induced OT-I proliferation is TAP independent in vivo*

To evaluate the potential of *Lm* NT-OVA to serve as a model Ag to study cross-presentation in vivo, B6.SJL congenic or TAP KO mice received i.v. 2–5 × 10^6 CFSE-labeled OT-I CD8$^+$ T cells, and were subsequently inoculated intradermally with 10^5 *Lm* NT-OVA or 3′NTs metacyclics, 5 μg of SIINFEKL peptide, or saline. DLNs were isolated 5 days later, and the intensity of CFSE fluorescence was determined on tetramer$^+$TCRβ^+CD8$^+$ OT-I T cells. Comparable cross-presentation of NT-OVA in *Lm* NT-OVA-infected B6.SJL and TAP KO mice was observed, with 62 and 50%, respectively, of tetramer$^+$TCRβ^+CD8$^+$ gated, transferred OT-I cells showing a reduced CFSE content (Fig. 4). Mice injected with control 3′NT parasites showed only background levels of OT-I cell proliferation (22 and 17%), comparable to saline controls. Injection of 5 μg of SIINFEKL peptide induced maximal proliferation with 99 and 98%, respectively, of OT-I T cells with reduced CFSE content.

The presentation of Lm *Ags by infected DC is TAP independent*

The involvement of TAP was also investigated in the context of the response of CD8$^+$ T cells primed to WT *Lm* Ags in vivo. CD8$^+$ T lymphocytes were purified from the spleen and DLNs of C57BL/6 mice 6 wk after intradermal inoculation with 10^4 *Lm* metacyclic promastigotes. The primed CD8$^+$ T cells were incubated with uninfected or *Lm*-infected WT or TAP KO DC, and levels of IFN-γ were measured in 72-h cell culture supernatants (Fig. 5*A*). Primed CD8$^+$ T cells responded to *Lm*-infected TAP KO or WT DC stimulation, with similar levels of IFN-γ ($p >$ 0.05). Background levels of IFN-γ were measured in cultures containing uninfected DC plus primed CD8$^+$ T cells, or DC, or infected DC alone. To confirm that CD8$^+$ T cells were the major source of IFN-γ, the cells were cultured with or without anti-CD8-depleting Ab (Fig. 5*B*). In the presence of anti-CD8, IFN-γ levels were reduced by 70% ($p = 0.0004$) and 88% ($p = 0.002$) in response to *Lm*-infected WT or TAP KO DC, respectively. In addition, only parasite-primed CD8$^+$ T cells, but not naive CD8$^+$ T cells, responded to *Lm*-infected WT or TAP KO DC.

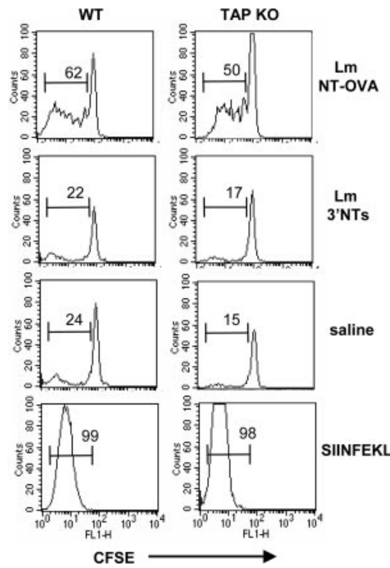

FIGURE 4. Adoptively transferred OT-I cells proliferate in response to *Lm* NT-OVA infection in the absence of TAP. CFSE-labeled OT-I CD8$^+$ T cells were injected i.v. into WT B6 or TAP KO recipient mice, which were subsequently challenged in the ear dermis with 10^5 *Lm* 3′NTs or NT-OVA, 5 μg of SIINFEKL peptide, or saline. CFSE fluorescence in the DLNs was measured 5 days later. Numbers represent percentage of tetramer$^+$TCRβ^+CD8$^+$ cells showing a reduced CFSE content. The experiment is representative of two separate experiments.

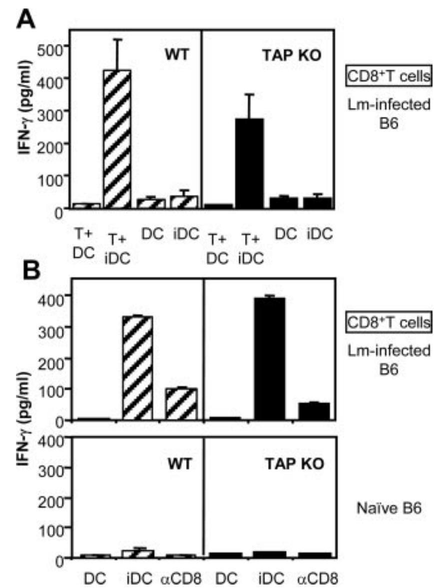

FIGURE 5. In vitro cross-presentation of *Leishmania* Ags to primed CD8$^+$ T cells does not require TAP. *A*, CD8$^+$ T cells from 6- to 8-wk *Lm*-infected B6 mice were positively selected by magnetic bead separation and incubated with uninfected or *Lm*-infected WT (▨) or TAP KO mice (■). *B*, Response of CD8$^+$ T cells from naive or *Lm*-infected B6 mice to infected WT and TAP KO DC, and effect of depleting anti-CD8 mAb. Levels of IFN-γ in 72-h cell culture supernatants were measured by ELISA. The data shown are the mean ± SD of three replicates, and the experiment is representative of two separate experiments.

TAP KO mice maintain CD8$^+$ T cell-dependent resistance to Lm

CD8$^+$ T cells have been shown to play an important role in the control of *Lm* infection in C57BL/6 mice (13). We further addressed whether TAP KO mice would also mount a protective, CD8$^+$ T cell-dependent, immune response to *Lm* infection. C57BL/6 and TAP KO mice were inoculated with 10^4 *Lm* promastigotes in the ear dermis. Lesion scores were measured weekly (Fig. 6*A*), and the number of parasites in the lesion was determined at 4 and 10 wk postinfection (Fig. 6*B*). Lesions progressed during the first 6 wk postinfection, and while there was considerable variability in the onset of healing, by 14–16 wk, all mice in both groups demonstrated significant reduction in lesion scores, with no significant difference between WT and TAP KO mice (Fig. 6*A*). At 4 wk postinfection, an average of 1.6×10^5 parasites was present in the ear of TAP KO compared with 1.3×10^5 in C57BL/6 mice. At 10 wk, both strains showed a significantly reduced number of parasites per ear (95–96%), with $7.1 \pm \times 10^3$ and $4.6 \pm \times 10^3$ parasites, respectively, remaining in TAP KO ($p < 0.0001$) and C57BL/6 mice ($p = 0.015$) (Fig. 6*B*). The differences in parasite loads between mouse strains at 4 and 10 wk postinfection were not statistically different ($p = 0.80$, $p = 0.55$). To further confirm the involvement of CD8$^+$ T cells in the control of infection by TAP KO mice, animals were infected intradermally with 10^4 *Lm* promastigotes and injected i.p. weekly with 1 mg/ml depleting anti-CD8 (clone 2.43) or control IgG (clone GL113) mAb. At 8 wk postinfection, TAP KO mice treated with anti-CD8 mAb harbored 4.1×10^4 parasites per ear compared with 1.5×10^3 in the control group ($p = 0.0003$) (Fig. 6*C*). The prolonged treatment with anti-CD8 mAb typically resulted in a 10-fold, although still incomplete reduction in total CD8$^+$ T cells present in DLNs and spleen when the mice were sacrificed 8 wk postinfection (data not shown).

Naive TAP KO mice have on average 10-fold (85%) less CD8$^+$ T cells in the ear, lymph nodes (LNs), and spleen compared with WT C57BL/6 mice. Inoculation of TAP KO mice with *Lm* resulted in a strong infiltration of mononuclear cells at the site of infection in the ear, with a 7- to 12-fold increase in effector memory CD44$^+$CD8$^+$ T cells (Fig. 7*A*). In addition, >80% of ear CD8$^+$ T cells stained positive for granzyme B and CD107a, and 13% responded to PMA/ionomycin stimulation by secreting IFN-γ, all indicators of effector functions (Fig. 7*B*). At wk 10–12 postinfection, the difference in numbers of CD8$^+$ T cells in the ear was reduced to 1.9-fold between TAP KO and C57BL/6 mice. An increase in effector CD8$^+$ T cells was also observed in spleen and LNs of *Lm*-infected TAP KO mice compared with naive mice, with 26% staining positive for IFN-γ, 25% for TNF-α (data not shown), 66% for granzyme B, and 27% for CD107a. A modest increase in total CD8$^+$ T cell numbers in the spleen (1.4-fold) and DLNs (2-fold), respectively, was also observed (data not shown). Finally, the Ag specificity of primed CD8$^+$ T cells from *Lm*-infected TAP KO mice was addressed by in vitro stimulating purified splenic and LN CD8$^+$ T cells with infected DC from either WT or TAP KO mice. CD8$^+$ T cells from infected TAP KO mice released IFN-γ in response to *Lm*-infected WT and TAP KO DC, but not uninfected DC (Fig. 7*C*). In addition, in the presence of anti-CD8 mAb, IFN-γ levels were reduced by 57% ($p = 0.0001$) and 70%, respectively ($p = 0.0034$), in response to infected WT or TAP KO DC. Background to low levels of IFN-γ were detected when naive CD8$^+$ T cells from TAP KO mice were cultured with *Lm*-infected DC. Thus, *Lm*-specific CD8$^+$ T cells essential for the control of cutaneous leishmaniasis are effectively primed in TAP KO mice.

Discussion

Many intracellular pathogens that are confined within host cell phagosomes are able to induce CD8$^+$ T cells in vitro and in vivo,

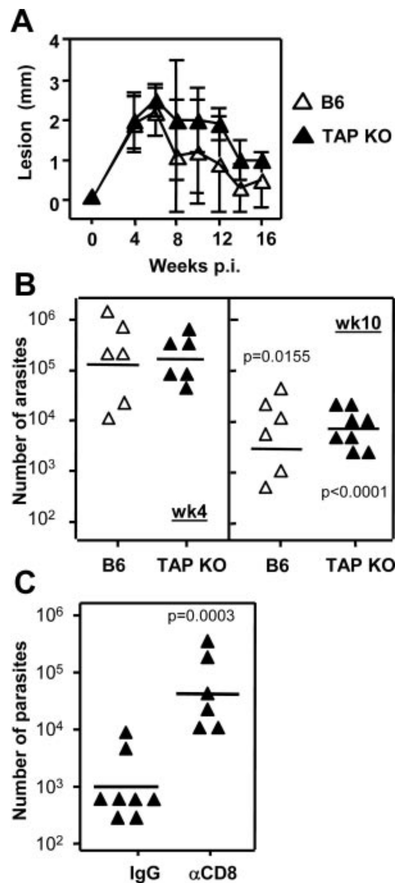

FIGURE 6. TAP KO mice maintain CD8$^+$ T cell-dependent resistance to *Lm* infection. B6 (open symbols) or TAP KO (filled symbols) mice were infected in the ear dermis with 10^4 *Lm* promastigotes. *A*, Development of lesions was monitored weekly. *B*, Ear tissue homogenates were titrated to quantify the number of parasites at 4 and 10 wk postinfection. *C*, Parasite burden in the ear of TAP KO mice treated i.p. weekly for 8 wk with 1 mg/ml anti-CD8 (clone 2.43) or control IgG (clone GL113) Abs. The experiment is representative of three separate experiments.

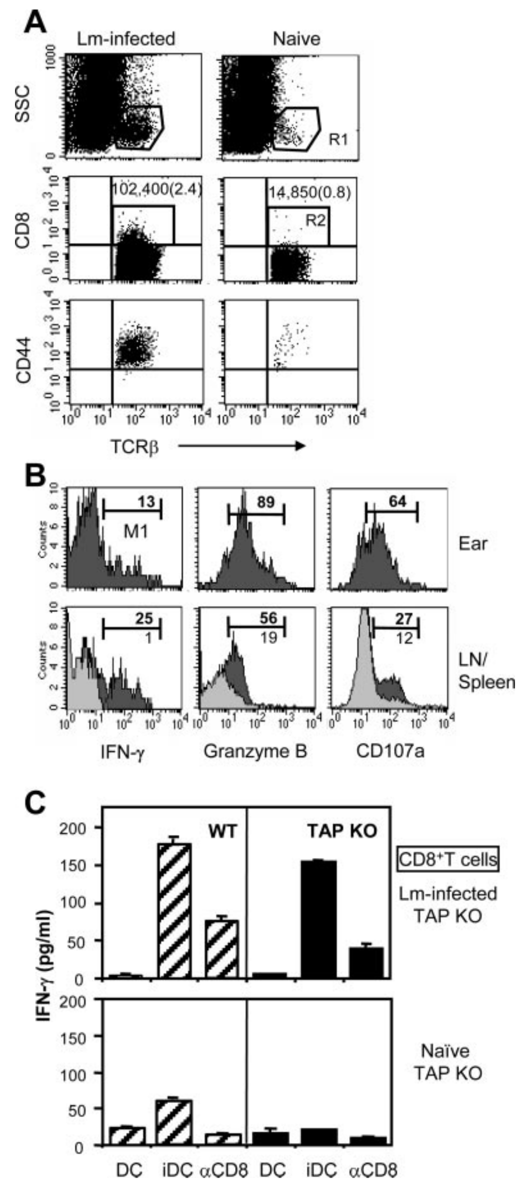

FIGURE 7. Characterization of CD8$^+$ T cells primed in vivo in TAP KO mice during *Lm* infection. TAP KO mice were infected in the ear dermis with 10^4 *Lm* promastigotes for 10–12 wk. *A*, Recruitment of activated CD8$^+$ T cells in the ear. Absolute numbers (percentage) of TCRβ^+CD8$^+$ cells per ear. *B*, Effector functions of ear and LN/spleen CD8$^+$ T cells from *Lm*-infected (dark histograms) or naive (light histograms) mice. *C*, Ag-specific response of CD8$^+$ T cells from *Lm*-infected or naive TAP KO mice to infected WT (▨) or TAP KO DC (■), and effect of anti-CD8 mAb. Levels of IFN-γ in 72-h cell culture supernatants were measured by ELISA. The data shown are the mean ± SD of three replicates, and the experiment is representative of two separate experiments.

and in the case of *Leishmania*, this response is required for protection. Nonetheless, the mechanisms controlling the cross-presentation of phagosome-derived Ags are poorly understood. The presentation of the SIINFEKL peptide by *Lm* NT-OVA-infected DC in vitro or by APC involved in cross-priming in vivo was not affected by the absence of TAP, suggesting that the conventional MHC class I processing pathway is not necessary and may not be used for this particular epitope. This conclusion was further supported by the observations that the proteasome was dispensable, while endosomal protease activity was required for optimal cross-presentation of *Lm* NT-OVA to proceed. When we extended these studies to the cross-presentation of WT *Lm* Ags recognized by in vivo primed CD8$^+$ T cells, a dispensable role for TAP was still observed. Most importantly, the priming of CD8$^+$ T cells involved in acquired resistance to *Lm* was not compromised by the absence of TAP. Thus, in the case of *Lm*, cross-presentation does not appear to follow a phagosome-to-cytosol pathway as has been reported for other intracellular microorganisms for which TAP dependency has been examined, including *M. tuberculosis* (36, 37), *L. monocytogenes* (20, 38), *E. coli* (34, 39), or *Streptoccocus gordonii* (40). In the current studies, the presentation of OVA by *Tg* P30-OVA-infected DC was strictly TAP and proteasome dependent, consistent with the findings of Gubbels et al. (41). In contrast, the requirement for TAP in the processing of OVA or rNT-OVA originating within model latex bead phagosomes could be bypassed with higher concentrations of Ag, consistent with the greater efficiency that has been reported for TAP-dependent cross-presentation in vitro and in vivo (23, 33–35, 42). Thus, avoidance or inhibition of the more efficient, TAP-dependent cross-presentation machinery by Ags sequestered within the *Leishmania* phagosome may represent a strategy whereby the parasite can delay the onset of CD8$^+$ T cell priming, as has been clearly observed in experimental mouse models of *Lm* infection (13, 43).

The comparison of OVA cross-presentation following uptake of *Lm* NT-OVA vs NT-OVA-latex beads is especially instructive as

both Ags are released from within mature endocytic compartments (44). The ability of Ags derived from latex beads to be targeted through a phagosome-to-cytosol pathway is clear from a number of studies that have documented the TAP and/or proteosome requirements for cross-presentation of OVA originating within latex bead phagosomes (16–18, 45, 46). Our studies do not directly address the current controversy (47, 48) regarding the role of ER-mediated phagocytosis in cross-presentation, which postulates that the ER-based machinery required for egress of Ags into the cytosol for proteosomal processing and their subsequent transport via TAP back into the phagosome for loading onto MHC class I become directly integrated into the endocytic pathway (16–18). The emphasis of the current findings is that whatever mechanisms may account for the fact that phagosomes associated with latex beads and most other intracellular pathogens permit targeting of Ags to the cytosolic pathway, the *Leishmania* phagosome appears to lack one or more elements of this pathway. This conclusion is based on the inability of the TAP-sufficient DC infected with *Lm* NT-OVA to activate OT-I cells earlier or at lower MOI compared with the DC lacking TAP, as would be predicted from the greater efficiency of the TAP-dependent mechanisms. The fact that *Lm*-infected, TAP-deficient mice were not compromised with respect to *Lm*-specific $CD8^+$ T cell priming and $CD8^+$ T cell-dependent acquired resistance suggests that WT *Lm* Ags also fail to be targeted through a properly constituted, ER-based processing pathway by APC in vivo.

The TAP-independent processing of *Leishmania* Ags suggests that their cross-presentation relies on the generation of peptide ligands and post-Golgi loading of MHC class I molecules within the phagosome, as proposed for other model exogenous Ags (49–52). Direct demonstration that the TAP-independent cross-presentation of Ags delivered by microparticles relies on peptides generated in the phagosome, and the involvement of cathepsin S in this process, has recently been reported (35). Neutralization by chloroquine of the pH in DC phagosomes and inhibition of endocytic proteases by leupeptin reduced *Lm* NT-OVA processing by DC and TAP KO DC, while proteasome inhibitors had only minor effects, further supporting an endosomal processing of *Leishmania* Ag. These results are in contrast with the sudy of Kima et al. (53), who reported that the presentation of *Leishmania amazonensis* GP46/M-2 Ag by infected macrophages was following an endogenous pathway, sensitive to brefeldin A and proteasome inhibitors. It is unclear, however, whether the inhibitors used in their study were solely affecting the endogenous pathway, as no peptide control was provided. It is also possible that Ags from different *Leishmania* species follow different processing and presentation pathways. The presence of peptide/MHC class I complexes within the *Lm* phagosome also needs to be confirmed. The possibility that peptide ligands bind to surface MHC class I molecules following peptide regurgitation, as has been described (12, 23), can effectively be ruled out, because direct contact between the *Lm* NT-OVA-infected DC and OT-I cells was necessary to induce their proliferation (29).

The likely requirement for phagosome maturation/endocytic proteases to generate TAP-independent ligands may explain why DC infected with *Tg* P30-OVA, residing within vacuoles that do not fuse with endosomes/lysosomes, relied on cytosolic proteasome activity and were unable to use a TAP-independent pathway for OVA cross-presentation, even at high MOI. Although the TAP and proteasome dependency of the cross-presentation requires some form of vacuole-to-cytosol Ag transfer, as recently suggested (41), whether an ER-based retrotranslocon is involved remains to be determined. The involvement of an ER-based MHC class I-loading complex incorporating TAP in the transport of peptide ligands back into the *Toxoplasma* vacuole for loading onto MHC class I would also need to be confirmed.

DC appear capable of multiple phagosomal processing pathways, perhaps reflecting their encounter with intracellular pathogens such as *Leishmania* that have coevolved to inhibit or avoid the efficient phagosome-to-cytosol/ER-based cross-presentation pathway. Understanding these alternative MHC class I processing and presentation pathways may prove useful for appropriate targeting of exogenous vaccine Ags for efficient priming of protective $CD8^+$ T cell responses against *Leishmania* and other intracellular pathogens.

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Disclosures

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