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TAP-Dependent and -Independent Peptide Import into Dendritic Cell Phagosomes

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Cross-presentation of phagocytosed Ags by MHC class I (MHC-I) molecules is thought to involve transport of cytosolic peptides into dendritic cell phagosomes, mediated by TAP transporters recruited from the endoplasmic reticulum. However, because pure and tightly sealed phagosomes are difficult to obtain, direct evidence for peptide transport into phagosomes has remained limited. Moreover, the parameters determining peptide uptake by, and survival in, phagosomes remain little characterized. In this study, we monitored peptide import into phagosomes by flow cytometry using two types of fluorescent reporter peptides, one of which directly bound to intraphagosomal beads. We observed that a peptide with high TAP affinity is imported into phagosomes in a TAP- and ATP-dependent manner, as expected. However, surprisingly, import of the OVA peptide SIINFEKL, a CD8+ T cell epitope frequently used to study cross-presentation, is ATP-dependent but substantially TAP-independent. The half-life of both reporter peptides is shortened by enhanced phagosomal maturation triggered by TLR signaling. Collectively, these results confirm that TAP can import peptides into phagosomes, but they suggest that some peptides, including the popular SIINFEKL, can enter phagosomes also via a second unknown energy-dependent mechanism. Therefore, the frequently reported TAP dependence of cross-presentation of phagocytosed OVA may principally reflect a requirement for recycling MHC-I molecules rather than SIINFEKL import into phagosomes via TAP. The Journal of Immunology, 2016, 197: 3454–3463.

Dendritic cells (DCs) can load MHC class I (MHC-I) molecules with peptides derived from phagocytosed Ags in a process known as cross-presentation (1). Among several pathways of cross-presentation described, a pathway implicating Ag degradation in the cytosol followed by import of peptide products into phagosomes is considered to play an important or even dominant role. Peptide access to phagosomes is thought to be mediated by the TAP transporters, a notion consistent with detection in phagosomes, using immunoblot and microscopy of multiple components of endoplasmic reticulum (ER) peptide-loading complexes, including TAP (2–4), and corroborated by Sec22b-dependent fusion of the ER–Golgi intermediate compartment with phagosomes (5).

An important argument supporting the concept of loading of phagosomal MHC-I with peptides produced in the cytosol has been the demonstration of TAP- and energy-dependent peptide transport in phagosomes. Various assays monitoring accumulation and/or MHC-I binding of labeled or glycosylated peptides have been used by multiple groups, including ours (2, 3, 5). However, the difficulty of ruling out a role for ER vesicles contaminating phagosomes can complicate interpretation of the results used in such assays. Moreover, these assays have generally used a very small number of reporter peptides with particularly high TAP (and/or MHC-I) affinity, so that the observations made may not easily be generalizable.

Another caveat complicating the interpretation of phagosomal peptide transport assays is contributed by the dual effect of TAP deficiency in cross-presentation assays. TAP knockout (KO) cells not only lack TAP as a putative mechanism for peptide import into phagosomes (and into the ER), but they also have greatly diminished numbers of MHC-I molecules on the cell surface. Given that recycling class I molecules are thought to be required for cross-presentation (6), the latter effect alone might explain poor cross-presentation by TAP KO DCs. We have recently observed that cross-presentation of phagocytosed OVA by TAP KO DCs could be restored by normalizing cell surface MHC-I levels through DC incubation at 26°C (7). As restored cross-presentation by TAP KO DCs remained proteasome-dependent, suggesting Ag degradation in the cytosol, one potential explanation of this phenomenon was the existence of multiple mechanisms for peptide import into phagosomes.

Motivated by these observations, in this study we tested the model of TAP- and ATP-dependent phagosomal peptide transport using different reporter peptides. We also asked what parameters affected peptide accumulation and survival in phagosomes. Our results are consistent both with the role of TAP as a phagosomal peptide transporter and with the existence of a second active and peptide-selective mechanism for peptide import into phagosomes.

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Abbreviations used in this article: BMDC, bone marrow–derived DC; DC, dendritic cell; ER, endoplasmic reticulum; KO, knockout; MHC-I, MHC class I; qPCR, quantitative PCR; R9L, peptide RRYNASTEL; S8L, peptide SIINFEKL; WT, wild-type.

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Materials and Methods

Mice and cells

C57BL/6 and BALB/c mice were purchased from Janvier (Le Genest-Saint-Isle, France). TAP1-deficient C57BL/6 mice (8) and NOD mice were maintained in our animal facility. To produce DCs, the long bones of mice were flushed with PBS. The cells obtained were resuspended at 0.5 × 10^7/ml in IMDM (Sigma-Aldrich, Saint-Quentin Fallavier, France) supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µg 2-ME (referred to hereafter as complete IMDM) (all supplements from PAA Laboratories, Velizy-Villacoublay, France), and 5% supernatant of J558 cells producing GM-CSF (9). Bone marrow-derived DCs (BMDCs) were used on day 6 or 7 for differentiation for experiments.

Reagents

Unless indicated otherwise, laboratory reagents were purchased from Sigma-Alrich; these include polyoamine, creatine kinase, creatine phosphate, and LPS 055:BS from Escherichia coli. Beads used for phagocytosis were either 3-µm polybeads amino or 2-µm streptavidin microspheres, both from PolyScience (Niles, IL). EDTA-free cOmplete protease inhibitor cocktail tablets were from Roche Diagnostics (Meylan, France). CellMask Deep Red, a reagent staining membrane phospholipids, was from Invitrogen (Cergy Pontoise, France). An FITC-labeled rat mAb recognizing murine CD107b (Lamp-2b; clone ABL-93) was obtained from BD Biosciences, a FITC-labeled mAb against rat Ig was from SouthernBiotech, and PE-labeled goat Abs against mouse IgG were from Beckman Coulter. The hybridoma-producing mAb 25D1 recognizing SIINFEKL-K^c complexes was a gift of M. Princicotta (10).

Peptides

Peptide RRYNAC(FITC)TEL (R9L-FITC) was obtained with >80% purity from Pepscan (Lelystad, the Netherlands), whereas SIINFKEK(FITC)K (SLI-FITC) >80% purity was from JPT Peptide Technologies (Berlin, Germany). The peptides RK(biotin)YNAC(FITC)TEL (R9L-Bio-FITC) and SIIN(FITC)K (SLI-Bio-FITC) were obtained >95% pure from Biosyn (Berlin, Germany). Dy490 is a fluorochrome analog of FITC/Alexa Fluor 488. Unlabeled peptides RRYNASTEL (R9L) and SIINFEKL (S8L) were >80% pure and obtained from Schafer-N (Copenhagen, Denmark).

Preparation of phagosomes

Three-micrometer polybead amino latex beads were activated by incubation for 4 h in 8% glutaraldehyde at 25°C, washed twice with cold PBS, and then coated overnight at 4°C with 100 µg/ml purified rabbit Ig in PBS. Coupling was stopped by adding 0.2 M glycine for 30 min at room temperature. Ig-coated polybeads, or streptavidin-coupled beads, were added at a ratio of three beads per cell to BMDCs suspended in IMDM at 37°C without brake. When the effect of phagosome maturation on peptide transport was examined using unlabeled reporter peptides, peptides R9L-FITC or S8L-FITC were added at 5 µM. Where indicated, an excess of unlabeled competitor peptides at various concentrations was added at the same time. Then the phagosomes were incubated for 15 min at 20 or 4°C. Peptide transport was finally incubated for 30 min at 4°C with 7.5 µg/ml CellMask in PBS. Peptide accumulation in phagosomes was examined using BD FACScalibur or FACSCanto II equipment (BD Biosciences). Phagocytosed beads were identified using sequential gating in a forward scatter/side scatter dot plot (as defined by running free beads) and as a FL4 (CellMask)-positive population. FACS data were analyzed using FlowJo 9.6.2 software (FlowJo, Ashland, OR).

When biotin and fluorochrome double-labeled reporter peptides were used, the assay was performed in the same manner, but with the following modifications. Reporter peptides R9L-Bio-FITC and S8L-Bio-Dy490 were added at 2 nM, and the vesicles were incubated for 15 min at 25°C. Peptide transport was terminated by washing in cold PBS followed by membrane lysis by addition of PBS containing 1% BSA and 1% SDS, bead centrifugation at 3000 × g, and repeated washing in PBS. CellMask was not used for SDS-treated beads. Beads were identified and analyzed as described for assays with single-labeled reporters.

Phagosome staining for Lamp-2b

Early and late phagosomes were permeabilized by incubation for 10 min at 4°C with a solution of 1% paraformaldehyde in PBS. Permeabilized phagosomes were incubated for 1 h at 4°C with 2% BSA in PBS to block nonspecific Ab-binding sites. Then the vesicles were stained by a 30-min incubation at 4°C with a FITC-conjugated rat mAb recognizing Lamp-2b (5 µg/ml) followed by washing and another 30-min incubation with an FITC-conjugated mouse mAb recognizing rat Ig (5 µg/ml). The phagosomes were finally stained with CellMask and analyzed by cytometry as described above.

Phagosome staining using mAb 25D1

Phagosomes were subjected to parallel standard transport assays using S8L-FITC and unlabeled peptide S8L as reporter peptides. After terminating peptide transport, the phagosomes were permeabilized using 0.05% saponin in PBS for 3 min at 4°C before incubation with 10 µg/ml mAb 25D1 (10) dissolved in PBS with 0.05% saponin for 30 min at 4°C, followed by simultaneous staining with secondary PE-labeled goat Abs against mouse IgG (diluted 1:100) and CellMask.

Peptide binding to TAP complexes

Peptide binding to cell microsomes expressing murine TAP1 or TAP2, or TAP1/2 complexes, was measured exactly as described in Burgevin et al. (11), using [125I]-labeled reporter peptide R9L and serially diluted competitor peptides.

Lentiviral TAP2 knockdown

Plasmid pLKO.1-puro carrying a short hairpin RNA sequence specific for murine TAP2 (TRCN00000066389, 5'-CCGG-GCTAC-AAGGA-TCTCT-CAGA-NCCTCAGGTTCCTAG-TTAGTT-CCTTT-GTTTG-3') and control plasmid carrying a nontargeting sequence (SHC002H), both with a puromycin resistance gene, were purchased from Sigma-Aldrich. The pLKO.1 plasmids were cotransfected with the packaging plasmids pCMVDeluxa2.2 and the envelope plasmid pMD2G into HEK-293-FT cells via calcium chloride transfection. Twenty-four hours later the buffer was exchanged for complete DMEM and virus-containing supernatant was collected 48, 72, and 96 h posttransfection. The supernatants were concentrated by spinning at 22,000 rpm for 2 h at 4°C and removal of medium. Viral pellets were resuspended in 100 µl of medium overnight at 4°C on an orbital shaker. To determine virus titers, bone marrow cells at day 2 of culture in the presence of GM-CSF and supplemented with 8 µg/ml Polybrene were transduced by centrifugation for 90 min at 37°C with several dilutions of viral supernatants. On day 4 of culture, cells were treated with 5 µg/ml puromycin, and on day 7, total RNA was prepared by the RNAspin mini kit (GE Healthcare). cDNA was obtained using the ImProm-II reverse transcription system (Promega) with random hexamers and 1 µg of total RNA. Quantitative PCR (qPCR) was performed with the SYBR Green method.

Peptide transport assay

Crude phagosomes purified as described above were incubated for 5 min at 20°C in PBS containing 900 µM ATP, 200 µM glycine, and 40 mM creatine phosphate (ATP-regenerating system) or incubated for 15 min at 37°C with 10 µg/ml apyrase in PBS to deplete ATP. In the assay using single-labeled reporter peptides, peptides R9L-FITC or S8L-FITC were added at 5 µM. Where indicated, an excess of unlabeled competitor peptides at various concentrations was added at the same time. Then the phagosomes were incubated for 15 min at 20 or 4°C. Peptide transport was finally incubated for 30 min at 4°C with 7.5 µg/ml CellMask in PBS. Peptide accumulation in phagosomes was examined using BD FACScalibur or FACSCanto II equipment (BD Biosciences). Phagocytosed beads were identified using sequential gating in a forward scatter/side scatter dot plot (as defined by running free beads) and as a FL4 (CellMask)-positive population. FACS data were analyzed using FlowJo 9.6.2 software (FlowJo, Ashland, OR).

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Ag presentation assays

Presentation of OVA through the endogenous pathway was examined as described in Merzougui et al. (7), with the following modifications. BMDCs were infected for 4 h with trypsinized OVA-encoding vaccinia virus diluted in IMDM supplemented with 2.5% FCS. After fixation, neutralization, and washing of DCs, OT-I T cells were added at a ratio of 1:5:1 and in duplicates incubated for 24 h with DCs. Then, 10^{-10} M peptide S8L and an irrelevant peptide (SNYLFTKL) predicted to bind with high affinity to H-2Kb according to the SYFPEITHI algorithm (http://www.syfpeithi.de) were included in all assays as positive and negative controls, respectively. T cell stimulation was evaluated by IL-2 ELISA exactly as described (7).

Results

Identification of crude phagosomes

To measure peptide accumulation in phagosomes, we adopted the phagFACS assay (12) that analyzes crude phagosomes. BMDCs differentiated for 7 d were incubated for 20 min with Ig-coated latex beads, separated from free beads by two centrifugations over FCS layers and then broken up mechanically. To identify phagosomes, a forward/side scatter gate was established using free beads and applied to BMDC lysates (Fig. 1A). To exclude remaining contaminating free beads from analysis, phagosomes were stained with CellMask, a reagent binding membrane phospholipids (Fig. 1A). Exclusion of CellMask-negative beads removed 5–15% of beads from analysis (Fig. 1). CellMask staining was not affected by changes associated with phagosome maturation such as an acid pH or altered lipid composition, as acquisition of Lamp-2b staining, indicative of phagosome maturation, did not affect CellMask staining (Fig. 1B). CellMask staining was routinely used for gating of phagosomes in subsequent phagFACS experiments.

Specific accumulation of fluorescent reporter peptides in crude phagosomes

Asking whether peptide transport into crude phagosomes could be monitored, we incubated such phagosomes for 15 min at 20°C with the FITC-conjugated reporter peptide R9L and analyzed peptide accumulation by flow cytometry. R9L, a peptide with very high affinity for both human and murine TAP (11) that has frequently been used for measuring TAP affinity (11, 13), associated with phagosomes in the presence of an ATP-regenerating system, presumably reflecting accumulation in phagosomes (Fig. 2A). Accumulation was absent or strongly reduced when incubations were performed at 4°C, or when ATP was depleted using preincubation with apyrase, consistent with active peptide transport. Accumulation was inhibited by unlabeled autologous peptide in a concentration-dependent manner (Fig. 2A, 2C).

We next sought to establish whether accumulation could also be monitored for a second peptide. We chose to examine peptide S8L, an H-2Kb-restricted OVA epitope with moderate TAP affinity (14) that is frequently used to study endogenous as well as crosspresentation given the availability of a specific TCR-transgenic mouse strain (OT-1) and an efficient TCR-like mAb (25D1). Similar to R9L-FITC, S8L-FITC accumulated in phagosomes in a temperature and ATP-dependent manner, which could be inhibited by addition of autologous unlabeled competitor peptide (Fig. 2B).

Comparing competition of unlabeled R9L and S8L with the two FITC-labeled reporter peptides, we observed that R9L competed with superior efficacy against both reporters, a finding entirely consistent with its significantly higher affinity for the murine TAP transporter (11).

Effect of TAP deficiency on phagosomal peptide accumulation

DC phagosomes are thought to be equipped with TAP that is recruited from the ER and mediates peptide import from the cytosol (1). Consistent with this notion, TAP deficiency abrogated accumulation of R9L-FITC in phagosomes (Fig. 3A, 3C). Incubation at 26°C is known to at least partially restore MHC-I expression at the surface of TAP-deficient APCs (15). We have previously found that preincubation at 26°C also restored cross-presentation of a phagocytosed Ag by TAP-deficient DCs, a phenomenon with at least three, mutually nonexclusive mechanistic explanations: reconstitution at the cell surface of normal levels of MHC-I, able to recycle through phagosomes; stabilization of empty MHC-I dimers allowing for rebinding of peptide in the endocytic pathway (16); and temperature-dependent recruitment to phagosomes of an alternative peptide transporter (7). Preincubation of BMDCs at 26°C did not reconstitute accumulation of R9L-FITC in phagosomes, arguing against the latter scenario, at least for this peptide (Fig. 3A, 3C).

Next we analyzed the effect of TAP deficiency on accumulation of reporter S8L-FITC. Surprisingly, and in striking contrast to R9L-FITC,
TAP deficiency had no significant effect on S8L accumulation (Fig. 3B). This was highly reproducible in seven independent experiments (Fig. 3D). TAP-independent accumulation of S8L-FITC was temperature- and ATP-dependent and not affected when the density of MHC-I molecules on the surface of BMDCs was increased by preincubation at 26°C (Fig. 3B, 3D). In conclusion, using our modified phagoFACS assay, we observed association of two different peptides with crude phagosomes that was ATP-dependent in both cases, whereas TAP was required for accumulation of R9L but not of S8L, a peptide epitope frequently used to study MHC-I cross-presentation.

Specific formation of pMHC complexes following transport of fluorescent peptides

We considered possible explanations for the surprising finding that S8L-FITC associated with phagosomes in a TAP-independent manner. One possibility was that modification by FITC, linked to Lys in peptide position 7, modified the properties of S8L so that it could be transported by a different transporter that cannot transport unmodified S8L and therefore does not act in physiologic cross-presentation. We reasoned that a modification with a strong effect on transporter interaction might also affect peptide binding to MHC-I molecules. S8L binds to H2-Kb molecules expressed by both WT and TAP-deficient BMDCs used in the assays described so far. Stable binding of S8L to H2-Kb can be detected using the TCR-like mAb 25D1 (10). We compared formation of S8L-Kb complexes in WT BMDCs and TAP-deficient BMDCs used in the assays described so far.

Expected, ATP-dependent formation of 25D1-reactive complexes (data not shown). To demonstrate the specificity of 25D1 staining, we performed parallel assays with phagosomes from NOD BMDCs that lack H2-Kb molecules (Fig. 4C). As expected, ATP-dependent formation of 25D1-reactive complexes was not observed in these vesicles. Additionally, accumulation of labeled S8L-FITC was detectable only in vesicles from acidd-stripped cells and even in this case was weak, suggesting a role of S8L-FITC binding to H2-Kb in detection of peptide association with phagosomes.

Although these experiments suggested that FITC conjugation does not affect H2-Kb binding by S8L, it was still possible that TAP transport was affected, for example such that S8L-FITC lost affinity for TAP1/2 complexes. TAP affinity can be assessed by measuring initial peptide binding to the transporter overexpressed in insect cell microsomes (11). We compared competition of unmodified and FITC-labeled peptides R9L and S8L for binding of iodinated reporter peptide R9L to microsomes expressing murine TAP1/2 complexes or single TAP subunits. As expected, the reporter peptide bound exclusively to TAP1/2 complexes and not to single TAP subunits (Fig. 5A). Consistent with our previous findings (11), the high-affinity peptide R9L competed more efficiently...
than did S8L. FITC conjugation increased competition by both R9L and S8L; however, the hierarchy of competition efficiency was not altered. Therefore, FITC conjugation does not affect TAP1/2 affinity of S8L in a manner distinct from R9L that would explain TAP independence of S8L contrasting with TAP dependence of peptide R9L.

Because our TAP-binding assay only evaluates affinity for TAP1/2 complexes, we considered that FITC-conjugated S8L might use TAP2 monomers or homodimers alone to access TAP1 KO phagosomes. TAP1-deficient BMDCs express normal amounts of TAP2 mRNA (data not shown). To rule out a role of TAP2 in TAP1-independent phagosomal import of S8L-FITC, we knocked down TAP2 expression using a lentivirus. TAP2 expression levels in WT and TAP1 KO BMDCs transduced with this lentivirus were reduced by 70% relative to cells transduced with a nontargeting virus, as determined by qPCR (data not shown) (Abs to murine TAP2 are not available). BMDCs transduced with the TAP2-specific lentivirus showed a 30–60% reduction in presentation of endogenous vaccinia virus–encoded OVA when tested with S8L-specific OT-I T cells (Fig. 5B), demonstrating partially effective knockdown of TAP2 expression. However, TAP2 knockdown had no effect on peptide import into TAP1 KO phagosomes, further confirming that TAP1/2 complexes are not required for import.

Taken together, the experiments shown in Figs. 4 and 5 suggest that TAP1-independent transport of S8L-FITC is not due to biochemical modification of the peptide altering peptide affinity for TAP, or due to transport by TAP2 alone, and they demonstrate that TAP is not required for peptide accumulation in phagosomes or for formation of S8L-Kb complexes presumably located in them.

Effect of phagosomal pH and binding to MHC-I on peptide accumulation

Next to the efficiency of peptide import into phagosomes, the duration of peptide survival in them is likely to modulate cross-presentation. Relative to macrophages and neutrophils, DCs are known to delay phagosome maturation associated with acidification and recruitment and activation of hydrolases so as to protect antigenic material from premature destruction (1). Indeed, accumulation and survival of R9L-FITC and S8L-FITC increased between 20 and 120 min in phagosomes from WT BMDCs (data not shown). Moreover, addition of a mixture of protease inhibitors during the transport assay did not increase peptide accumulation (Fig. 6A, 6B, two left-hand plots), suggesting that 20-min phagosomes of WT BMDCs harbor little activity capable of degrading peptides, consistent with published concepts (1). TLR signaling has been reported to activate phagosome maturation and MHC-II Ag presentation (17). Moreover, according to recent results, it can also enhance recruitment of MHC-I to phagosomes (6). Preincubation of BMDCs with a TLR4 ligand before and during phagocytosis strongly and indiscriminately reduced accumulation of both reporter peptides in 20-min phagosomes (Fig. 6A, 6B). However, consistent with enhanced recruitment and activation of hydrolases following TLR4 signaling, addition of protease inhibitors during transport assays with phagosomes from LPS-stimulated cells strongly increased peptide accumulation, although it did not restore it fully. Similar results were obtained with the TLR2 ligand Pam3Cys (data not shown).

The results shown in Fig. 4 (compare Fig. 4A and 4C) suggested that accumulation of peptide S8L in phagosomes was favored by the presence of H2-Kb, either by preventing peptide escape from the

**FIGURE 3.** Effect of TAP deficiency on phagosomal peptide accumulation. In (A), phagosomes from WT and TAP1-deficient BMDCs were incubated for 20 min in the presence of the ATP-regenerating system at 4 or 20˚C, or in the presence of apyrase. TAP-deficient BMDCs were either cultured at standard conditions (center rows) or incubated at 26˚C for 16 h (bottom rows) prior to phagocytosis at 37˚C. The panels in (B) show an equivalent experiment using S8L-FITC as reporter peptide, and (C) and (D) show the mean values ± SEM, normalized with respect to WT phagosomes, obtained in seven experiments set up as in (A) and (B), using R9L-FITC and S8L-FITC as reporter peptides.
vesicles or by protecting it from degradation. To confirm this hypothesis, we compared the effect of H2-K\textsuperscript{b} on accumulation of R9L-FITC and S8L-FITC. As shown in Fig. 6C, H2-K\textsuperscript{b} as expected increased accumulation of S8L-FITC but had no effect on that of R9L-FITC, which was unable to bind to H2-K\textsuperscript{b}. Moreover, acid stripping prior to phagocytosis, found to increase accumulation of S8L (see Fig. 4), had no effect on accumulation of R9L-FITC (data not shown). Taken together, the experiments in Fig. 6 demonstrate that TLR4 signaling increases the proteolytic activity but does not induce recruitment to phagosomes of H2-K\textsuperscript{b} in amounts sufficient to counteract increased proteolysis, thus arguing against significant recruitment of H2-K\textsuperscript{b} to phagosomes after 20 min.

A phagosomal transport assay directly monitoring intraluminal peptide accumulation

Similar to other assays published previously, the phagoFACS transport assay used so far is unable to distinguish between peptide accumulating within phagosomes and peptide in other vesicles, including ER copurifying with phagosomes. To overcome this limitation and measure exclusively peptide within phagosomes, we developed a novel assay using streptavidin-conjugated beads and reporter peptides labeled both with biotin and with FITC or the analog dye Dy490. In this assay, phagosomes were lysed with SDS and washed before FACS analysis, so that nonspecific background due to reporter binding to membranes or accumulating in contaminating ER was eliminated.

To establish experimental conditions, we first determined that the double-labeled peptides saturated streptavidin binding sites on free beads at 5 nM (data not shown), so that a concentration of 2 nM was chosen for transport assays. Next, we compared accumulation of single- and double-labeled reporter peptides in phagosomes containing Ig-coated beads (Fig. 7A). Transport of biotinylated peptides was temperature- and ATP-dependent as observed for single-labeled reporters. SDS lysis eliminated binding of both peptides, including ER copurifying with phagosomes. To overcome this limitation and measure exclusively peptide within phagosomes, we developed a novel assay using streptavidin-conjugated beads and reporter peptides labeled both with biotin and with FITC or the analog dye Dy490. In this assay, phagosomes were lysed with SDS and washed before FACS analysis, so that nonspecific background due to reporter binding to membranes or accumulating in contaminating ER was eliminated.

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confirming efficient membrane lysis and absence of nonspecific binding to latex beads (Fig. 7A). Conversely, when streptavidin-conjugated beads were used for phagocytosis, reporter peptide association with beads was eliminated by membrane lysis for single-labeled peptides but not for double-labeled reporter peptides (Fig. 7B). To establish whether the double-labeled reporter peptides entered phagosomes by a mechanism that could be saturated, we examined competition by unlabeled homologous peptides for reporter accumulation on phagosomal streptavidin-conjugated beads (Fig. 7C). Unlabeled competitor peptides indeed inhibited reporter accumulation on beads in a dose-dependent manner; however, competition was clearly less efficient than in the case of single-labeled reporter peptides.

Finally, we used the biotin-based assay to verify the TAP dependence of R9L and S8L transport into phagosomes. Binding to phagosome-enclosed beads of R9L(Bio)-FITC was temperature- and ATP-dependent and required expression of TAP (Fig. 8A). In contrast, import of S8L(Bio)Dy490 into TAP-deficient phagosomes, although still being temperature- and ATP-dependent, was only slightly reduced in comparison with WT phagosomes (Fig. 8B). Thus, results obtained with the assay measuring direct intraphagosomal peptide accumulation confirmed that transport of R9L was strictly TAP-dependent whereas that of S8L was not.

The mechanism of S8L import into TAP-deficient phagosomes remains unclear. We reasoned that, as a first step toward identifying it, comparing competition of unlabeled peptides for import into WT and TAP-deficient phagosomes might reveal different selectivity of the unknown mechanism relative to TAP selectivity. To study this, we used single-labeled S8L reporter peptide. As shown in Fig. 8C, unlabeled R9L and S8L competed for transport of S8L-FITC both in WT and TAP-deficient phagosomes in a dose-dependent manner. However, competition by both peptides was less efficient in the latter vesicles. Moreover, in contrast to the clear competition hierarchy observed in WT vesicles based on the higher TAP affinity of R9L, the two competitors were equally efficient in TAP-deficient vesicles, consistent with distinct selectivity of peptide import into WT and TAP KO phagosomes.

Discussion
We have employed two simple but nevertheless novel assays to examine the role of TAP in peptide import into DC phagosomes. Measuring peptide association with phagosomes, especially crude vesicles as used in this study, can be subject to several confounding phenomena and artifacts, including peptide import into contaminating ER, peptide binding to (phagosomal or other) membranes, nonspecific interactions of reporter peptides due to chemical modifications, and peptide binding to MHC-I molecules in contaminating ER, other membranes, and inside-out vesicles produced during cell breakup. Our assay using double-labeled peptides binding directly to intraphagosomal, SDS-treated beads should exclude many of these factors, including effects of MHC-I molecules and contaminating membranes and/or ER, but it does not rule out that reporter peptide labeling by FITC results in transport by a mechanism not acting on physiologic unlabeled peptides. However, equally efficient formation of H2-Kb complexes, as detected by mAb 25D1 (Fig. 4), with FITC-labeled and unlabeled peptide S8L argues against this notion. We also demonstrate that FITC labeling, although slightly increasing affinity of both R9L and S8L for TAP1/2 complexes, does not abrogate specific S8L binding to TAP complexes or alter the TAP affinity of S8L relative to R9L. Thus, the two biologically relevant interactions of peptides during import into phagosomes, which engage
TAP and MHC-I, are not altered by FITC labeling, validating use of our assay for monitoring of physiologic transport. However, the FACS profiles of peptide accumulation that appear as shoulders of the negative control peaks suggest that the assay sensitivity is limited such that the amount of peptide presented in most phagosomes is below the detection limit of a standard FACS analysis.

FIGURE 6. Effect of phagosomal pH and MHC-I binding on peptide accumulation. In (A) and (B), BMDCs were incubated for 100 min prior to phagocytosis and during it with LPS or solvent only. After a 20-min phagocytosis period, a transport assay was performed in the presence of a mixture of protease inhibitors or in its absence. Finally, accumulation of reporter peptides S8L-FITC (A) and R9L-FITC (B) in CellMask-positive phagosomes was evaluated by cytometry. The panels in (C) show the accumulation of fluorescent reporter peptides in phagosomes from C57BL/6 (H2-K^d) or BALB/c (H2-K^d) BMDCs following transport assays in the presence of the ATP-regenerating system or apyrase. One representative out of three experiments is displayed.

FIGURE 7. Assay for capturing transported peptides on intraphagosomal beads. In (A), C57BL/6 phagosomes containing Ig-coated beads were subjected to a 20-min transport assay in the presence of the ATP-regenerating system or apyrase, using either FITC-labeled (S8L-FITC, R9L-FITC), or double biotin and FITC-labeled reporter peptides. At the end of the transport period, the phagosomes were either washed as usual and CellMask stained, or lysed using a buffer containing 1% SDS before analysis by cytometry. (B) An experiment performed exactly as in (A), but here the phagosomes contained streptavidin-coated beads. The panels in (C) compare the capacity of unlabeled competitor peptides to inhibit accumulation of single- and double-labeled analogous reporter peptides during a standard 20-min transport assay. One representative out of three experiments is shown.
Using our assay and WT phagosomes, we made a number of observations consistent with the notion of TAP-dependent peptide import, such as ATP and temperature dependence as well as a hierarchy of competition by unlabeled peptides consistent with the known TAP affinities of peptides R9L and S8L (11). We also observed that TLR4 signaling inhibited peptide accumulation in phagosomes, at least partly due to activation and/or recruitment of proteases as documented by the effect of protease inhibitors. Coincidentally, the effect of LPS argues against a role of contaminating ER in the assay using single-labeled reporter peptides, because TLR4 signaling is not known to enhance proteolytic peptide degradation in the ER.

The principal biological result of this study evidently is the relative TAP independence of S8L transport. Although surprising, this result is not inconsistent with the observations made in our previous study of cross-presentation by TAP-deficient DCs (7). In that study we had speculated that the phagosomes of these cells might contain normal amounts of cross-presentable (OVA) peptide, as confirmed in this study, but lack MHC-I molecules that
would only be internalized in sufficient amounts after DC pre-incubation at 26˚C. Although we do observe a clear effect on peptide accumulation of presumably massive MHC-I internalization triggered by acid stripping (Fig. 4), DC preincubation at 26˚C had no significant effect (Fig. 3). However, this may have a kinetic reason; that is, whereas surface MHC-I molecules on DCs pre-incubated at 26˚C are internalized for many hours during an Ag presentation assay, the internalization period was limited to 20 min of phagocytosis in this study. Whatever the explanation for the lack of the 26˚C preincubation in the phagosome transport assay, our results strongly suggest that inefficient cross-presentation of phagocytosed OVA by TAP-deficient DCs is not due to lack of SIINFEKL peptide in phagosomes, leaving the shortage of MHC-I molecules as the most likely explanation.

The molecular identity of the TAP-independent mechanism for entry into phagosomes remains unclear. Note that this unknown mechanism may not be able to compensate for TAP deficiency fully, as suggested by the minor reduction in peptide accumulation of double-labeled S8L in TAP1 KO phagosomes observed in the stringent assay (Fig. 8B). Importantly, note that our data do not indicate that S8L cannot be transported by TAP—they only suggest that S8L can also enter phagosomes by an unknown second mechanism. With its broad specificity including the capacity to transport MHC-I ligands, the alternative TAP-L transporter is an evident candidate, although its lysosomal location argues against such a role (18). Moreover, R9L has been used as reporter to demonstrate peptide transport by TAP-L (19), rendering exclusive phagosomal transport of S8L but not R9L by TAP-L highly unlikely. It is surprising that R9L competes for transport of S8L-FITC by the unknown mechanism yet apparently cannot enter phagosomes itself through it. R9L may therefore act as a competitive antagonist for the unknown mechanism. Whereas in the case of TAP, such antagonists can be produced by adding long side chains to branched peptides (20), relatively small peptide modifications might be sufficient to convert a substrate to an antagonist for the unknown mechanism. The results in this study suggest that the mechanism giving access to S8L but not R9L is ATP- and temperature-dependent—like TAP transport. However, its specificity is different from that of TAP transport, because unlabeled S8L and R9L compete not only equally well but also less efficiently than in WT vesicles for peptide accumulation in TAP-deficient phagosomes. Future experimentation will show whether an unidentified transporter or another energy-dependent mechanism underlies specific and TAP-independent S8L import into phagosomes.

Disclosures

The authors have no financial conflicts of interest.

References


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