Tapasin−/− and TAP1−/− Macrophages Are Deficient in Vacular Alternate Class I MHC (MHC-I) Processing due to Decreased MHC-I Stability at Phagolysosomal pH

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Alternate class I MHC (MHC-I) Ag processing via cytosolic or vacuolar pathways leads to cross-presentation of exogenous Ag to CD8 T cells. Vacuolar alternate MHC-I processing involves phagolysosomal Ag proteolysis and peptide binding to MHC-I in post-Golgi compartments. We report the first study of alternate MHC-I Ag processing in tapasin−/− cells and experiments with tapasin−/− and TAP1−/− macrophages that characterize alternate MHC-I processing. Tapasin promotes retention of MHC-I in the endoplasmic reticulum (ER) for loading with high affinity peptides, whereas tapasin−/− cells allow poorly loaded MHC-I molecules to exit the ER. Hypothetically, we considered that a large proportion of post-Golgi MHC-I on tapasin−/− cells might be peptide-receptive, enhancing alternate MHC-I processing. In contrast, alternate MHC-I processing was diminished in both tapasin−/− and TAP1−/− macrophages. Nonetheless, these cells efficiently presented exogenous peptide, suggesting a loss of MHC-I stability or function specific to vacuolar processing compartments. Tapasin−/− and TAP1−/− macrophages had decreased MHC-I stability and increased susceptibility of MHC-I to inactivation by acidic conditions (correlating with vacuolar pH). Incubation of tapasin−/− or TAP1−/− cells at 26°C decreased susceptibility of MHC-I to acid pH and reversed the deficiency in alternate MHC-I processing. Thus, tapasin and TAP are required for MHC-I to bind ER-derived stabilizing peptides to achieve the stability needed for alternate MHC-I processing via peptide exchange in acidic vacuolar processing compartments. Acidic pH destabilizes MHC-I, but also promotes peptide exchange, thereby enhancing alternate MHC-I Ag processing. These results are consistent with alternate MHC-I Ag processing mechanisms that involve binding of peptides to MHC-I within acidic vacuolar compartments. The Journal of Immunology, 2003, 170: 5825–5833.

C onventional class I MHC (MHC-I) Ag processing involves Ags expressed in the cytosol of APC and does not deal with exogenous Ags. Professional APCs, however, do process exogenous Ags for presentation by MHC-I molecules to CD8 T cells. This mechanism has been termed alternate MHC-I Ag processing and forms the basis for cross-presentation of Ags and cross-priming of CD8 T cells (1–3). Alternate MHC-I processing plays an important role in the initiation of CD8 T cell responses (4, 5). A more complete understanding of alternate MHC-I processing is important to better understand host defense for infectious disease and to develop new vaccine strategies.

Alternate MHC-I processing has been reported in many systems and may proceed by distinct pathways (1, 2, 4, 6–9), including cytosolic and vacuolar processing mechanisms, with processing in different subcellular compartments. The cytosolic alternate MHC-I pathway involves transfer of exogenous antigenic proteins from vacuolar compartments to the cytosol, where they enter the conventional MHC-I processing and peptide loading pathway (3, 10–12). This mechanism is sensitive to proteasome inhibitors, brefeldin A (BFA; which blocks anterograde transport of nascent MHC-I through the Golgi complex and therefore its delivery to the cell surface) and deficiencies in TAP (3). The vacuolar alternate MHC-I pathway involves processing of Ags in vacuolar compartments (e.g., phagosomes or endosomes, without access to the cytosol) and binding of peptides to MHC-I molecules in post-Golgi vacuolar compartments (e.g., within phagosomes or possibly at the cell surface after recycling and regurgitation of peptide) (7, 13, 14). This pathway is resistant to proteasome inhibitors and short term treatments with BFA. In some experimental systems vacuolar alternate MHC-I processing is TAP-independent (5, 15–17), but in others it is partially inhibited by TAP deficiency due to a consequent decrease in expression of post-Golgi peptide-receptive MHC-I molecules (8, 9, 18).

Mutant cell lines and mouse strains that have mutations in components of the conventional MHC-I pathway have been instrumental for studying alternate MHC-I Ag processing. TAP is necessary for transport of peptides from the cytosol into the endoplasmic reticulum (ER) to bind MHC-I molecules (19–22). TAP-deficient cell lines and TAP1−/− mice are well characterized (19, 23), and cells from TAP1−/− mice have been used to study the influence of TAP on the expression of peptide-receptive MHC-I molecules that contribute to vacuolar alternate MHC-I processing (8, 9, 18). Tapasin is complexed with calreticulin, MHC-I-β2-microglobulin, and TAP, and is necessary for association of TAP with the other components of this peptide loading complex (24, 25). In addition to promoting assembly of the MHC-I peptide loading complex, tapasin has been suggested to help retain MHC-I molecules in the 

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Abbreviations used in this paper: MHC-I, class I MHC; BFA, brefeldin A; BMM, bone marrow-derived macrophages; CBS, citrate-buffered saline; ER, endoplasmic reticulum; PEM, peritoneal exudate macrophages; PeM, peritoneal macrophages.

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ER until they are loaded with high affinity peptides (25–32). Recently, tapasin−/− mice were described (33, 34). The present studies provide the first examination of alternate MHC-I Ag processing in tapasin−/− cells.

Macrophages from both TAP1−/− and tapasin−/− mice have MHC-I molecules that are poorly loaded, i.e., contain peptides that are not bound with high affinity. In TAP1−/− cells this is due to the absence of peptide transport into the ER, greatly reducing the number and diversity of peptides available for MHC-I binding. In tapasin−/− cells this may result from the absence of the complete peptide loading complex, and insufficient ER retention and peptide exchange to optimize peptide loading of MHC-I. Consequently, MHC-I molecules on TAP- or tapasin-deficient cells have a short half-life and low steady state expression at 37°C, but the stability and expression of these MHC-I molecules can be increased by incubation at 26°C (32, 35, 36). Expression of MHC-I on TAP-deficient cells is also restored by incubation with exogenous β2-microglobulin (8, 37, 38) (which enhances binding of peptide to MHC-I molecules) (39–41), expression of β2-microglobulin transgene (42), or incubation with exogenous stabilizing peptide (9, 19, 36). In addition to defects in conventional MHC-I Ag processing, TAP-deficient cells may have decreased levels of post-Golgi peptide-receptive MHC-I molecules that are needed for vacuolar alternate MHC-I Ag processing (8, 18, 38). These deficits are reversed by enhancing the expression of post-Golgi peptide-receptive MHC-I molecules by incubation at 26°C, addition of β2-microglobulin or addition of exogenous stabilizing peptide (8, 9, 18, 38).

In earlier studies (9) we investigated the role of previously bound peptides and their dissociation in generating peptide-receptive MHC-I molecules for use in alternate MHC-I Ag processing. TAP1−/− macrophages were incubated overnight with an initial exogenous peptide, producing a large cohort of peptide:MHC-I complexes that could influence subsequent peptide dissociation/exchange. Initial incubation with a stabilizing peptide enhanced rather than reduced subsequent binding and presentation of an exogenous readout peptide to T cells, implicating peptide dissociation/exchange. Furthermore, stabilizing peptides enhanced alternate MHC-I processing of HB101.CrI-OVA (Escherichia coli expressing an OVA fusion protein), indicating that peptide dissociation/exchange contributes to alternate MHC-I Ag processing.

The studies reported here investigated the impact of genetic deficiency in tapasin on alternate MHC-I Ag processing by macrophages. In addition, we compared the processing function of tapasin−/− and TAP1−/− macrophages to test the impact of altered availability, transport, and stability of post-Golgi MHC-I. In both tapasin−/− and TAP1−/− cells, MHC-I stability is decreased; in TAP1−/− cells, MHC-I transport to the cell surface is slowed, but in tapasin−/− cells, this transport occurs readily. We observed that alternate MHC-I processing of exogenous Ag expressed in bacteria was markedly suppressed in tapasin−/− and TAP1−/− macrophages relative to that in wild-type C57BL/6 macrophages. Nonetheless, presentation of exogenous peptide Ag, which can bind directly to MHC-I on the cell surface, was spared in tapasin−/− and TAP1−/− macrophages. In tapasin−/− and TAP1−/− cells, we propose that suboptimal peptide loading decreases the stability and function of MHC-I in the acidic vacuolar environment. Consistent with this hypothesis, acidic pH destabilized MHC-I molecules, particularly those expressed on tapasin−/− and TAP1−/− cells. Deficits in alternate MHC-I Ag processing and susceptibility of MHC-I to acidic pH were both reversed in TAP1−/− and tapasin−/− cells by incubation at 26°C. Our observations suggest that MHC-I presentation of peptides derived from exogenous Ag requires stable precursor peptide:MHC-I complexes that enter the phagosome, where acidic pH facilitates peptide exchange to form complexes of MHC-I with antigenic peptides from exogenous Ag.

Materials and Methods
Cells and Ags
Tapasin−/− mice (33) on C57BL/6 genetic background, TAP1−/− mice (23) on C57BL6 genetic background, and wild-type C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) were bred and housed under specific-pathogen-free conditions. Cells were cultured in standard medium consisting of DMEM (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated FCS (HyClone, Logan, UT), 5 × 10–6 M 2-ME, 1 mM sodium pyruvate, HEPES buffer, and penicillin/streptomycin (Life Technologies). Cells were incubated in a 5% CO2 atmosphere and, unless otherwise indicated, at 37°C. Activated peritoneal macrophages (24) were harvested by peritoneal lavage 4 days after i.p. inoculation with Con A (100 μg/mouse), incubated for 2 h in 96-well plates (105 cells/well), and washed to remove nonadherent cells. Bone marrow-derived macrophages (BMM) were derived from mouse femur marrow precursors cultured for 7–14 days in petri dishes in standard medium containing 20% conditioned medium produced by M-CSF-secreting LADMAC cells (43); murine IFN-γ (10 ng/ml; R&D Systems, Minneapolis, MN) was added for the last 24 h of this culture. BMM were detached by trypsin and a cell lifter (Corning-Costar, Corning, NY) and incubated in 96-well plates (105 cells/well) for 18 h in 10 ng/ml murine IFN-γ. The resulting cells were adherent with macrophage-like morphology, and >98% of the cells were CD11b positive by flow cytometry. The hybridoma CD80VA1.3 (2) was used to detect SiinFEKLKβ complexes. Kβ binding peptides included SiinFEKL (OVA257–264), KVRFDKL (OVA551–560), and FAPNYPAL (Sendai virus nucleoprotein324–333). Peptides were synthesized (Princeton Biomolecules, Langhorne, PA), dissolved in distilled water at 1 mM, and diluted to final experiment concentrations in standard medium. E. coli HB101.CrI-OVA constitutively expresses CrI-OVA, a fusion protein containing the SiinFEKL epitope (2), and was used as a particulate Ag to assess alternate MHC-I Ag processing. Bacteria were grown on Luria-Bertoni/ampicillin plates, resuspended in 10 mM MgCl2/0.04% glucose (OD520 = 1.0 for 2 × 108 bacterial/ml), and diluted in standard medium (1 × 106 HB101.CrI-OVA bacteria contain ~150 ng or 10–11 mol of CrI-OVA (2)).

Ag presentation protocols
Ag presentation experiments used either IFN-γ-activated BMM or PerC, which produced similar results in these studies. Macrophages were incubated in 96-well plates under various conditions before and during exposure to Ag, including incubation with BFA (2 μg/ml), with stabilizing peptide, or acidic buffer, or at 26°C. BFA was used to inhibit transport of nascent MHC-I molecules through the Golgi complex and to the cell surface (unless otherwise noted, BFA was added immediately before the addition of Ag and was present throughout the incubation with Ag). Exogenous peptide or HB101.CrI-OVA was added at various concentrations for 2 h with or without BFA (2 μg/ml). After addition of bacteria, plates were washed in 1200 × g to layer bacteria onto the macrophages. Macrophages were then fixed in 1% paraformaldehyde, washed, and incubated with T hybridoma cells (105/well) for 24 h. To determine IL-2 secretion by T hybridoma cells, culture supernatant (0.1 ml) was frozen, thawed, and cultured with 5 × 104 IL-2-dependent CTRL-2 cells (total volume, 0.15 ml) for 24 h (44), and 15 μl Alamar Blue (Alamar Biosciences, San Antonio, TX) was added for 18–24 h. Alamar Blue dye is reduced by metabolically active cells, shifting its relative absorbance at 550 nm (reduced and oxidized forms both have high absorbance at 550 nm, but only the oxidized form has high absorbance at 595 nm). Thus, IL-2-dependent CTRL-2 cell growth was assessed by subtracting OD595 from OD550. This product measured threshold sensitivity, plateau response, and IL-2 dose-response results similar to those of [methyl-3H]thymidine incorporation CTRL-2 proliferation assays. A minimum response was observed with culture of CTRL-2 cells with 0.004–0.04 U/ml recombinant murine IL-2 (Roche, Indianapolis, IN; 2 U/mg); the half-maximal response was seen with 0.04 U/ml IL-2, and a plateau response was produced by 0.4–4 U/ml IL-2. Ag processing assays were performed with triple-well plates. Each data point is expressed as the mean ± SD. In experiments involving exposure to acidic pH, macrophages were washed in citrate-buffered saline (CBS) titrated to various pH values and incubated in CBS plus BFA for various periods at room temperature under a standard atmosphere. For experiments in which SiinFEKL was present during acidic exposure, peptide was diluted in CBS at each pH and added to macrophages with BFA for 30 min at 37°C; macrophages were then washed and incubated with peptide for 1 h in standard medium. For experiments...
with subphysiological temperature, macrophages were incubated at 26°C for 18 h before the addition of Ag and throughout the Ag processing incubation.

**Flow cytometry**

BMM were replated in six-well plates (2 × 10⁶ cells/well) and incubated 18 h at 37°C with or without stabilizing peptide (FAPGNYPAL). From each well, the cells were detached with trypsin and a cell lifter, washed three times in FACS buffer (PBS containing 0.1% rabbit serum and 0.1 mg/ml BSA), and split into three wells of a round-bottom, 96-well plate. The cells were incubated for 30 min on ice with 5 μg/ml BSA), and split into three wells of a round-bottom, 96-well plate. Each well, the cells were detached with trypsin and a cell lifter, washed by incubation.

**Results**

Tapasin⁻/⁻ and TAP1⁻/⁻ macrophages are deficient in alternate MHC-I Ag processing, but still present exogenous peptide, suggesting a defect in MHC-I stability and function specific to the vacuolar processing environment

We tested alternate MHC-I Ag processing function of tapasin⁻/⁻ macropages relative to TAPI⁻/⁻ and wild-type macrophages. Macropages were incubated with bacterial Ag (E. coli HB101.Crl-OVA) to assess vacuolar alternate MHC-I Ag processing or with exogenous SIINFEKL peptide (OVA257–264) to assess peptide-receptive MHC-I at the cell surface (and possibly in post-Golgi vacuolar compartments to which peptide and MHC-I could target). HB101.Crl-OVA constitutively expresses the Crl-OVA fusion protein, containing the OVA sequence residues 257–277 fused to the bacterial cytoplasmic regulatory protein Crl (2). HB101.Crl-OVA is processed by macrophages via the vacuolar alternate MHC-I pathway for presentation of the OVA257–264-Kb (2, 8), which is recognized by CD80VA1.3 T hybridoma cells. Tapasin⁻/⁻ and TAPI⁻/⁻ macrophages were markedly deficient in alternate MHC-I Ag processing relative to wild-type C57BL/6 macrophages (with an ≈10-fold increase in the Ag dose required to match wild-type cells; Fig. 1, A and B). Nonetheless, all three types of macrophages presented exogenous SIINFEKL with similar efficiency. These results suggest that the defect in peptide loading of MHC-I in tapasin⁻/⁻ and TAPI⁻/⁻ macrophages decreases the survival and function of MHC-I in the vacuolar processing environment more than its ability to bind and present peptide at the cell surface.

**Alternate MHC-I Ag processing involves post-Golgi MHC-I molecules that have decreased stability on tapasin⁻/⁻ and TAPI⁻/⁻ macrophages**

To determine the role of nascent MHC-I molecules in alternate MHC-I processing in these systems, BFA and Ag (SIINFEKL or HB101.Crl-OVA) were added simultaneously to macrophages. After 2 h the cells were fixed and then incubated with CD80VA1.3 T hybridoma cells to determine the presentation of SIINFEKL-Kb complexes. Fig. 1 shows that acute exposure to BFA did not alter the binding and presentation of exogenous SIINFEKL (Fig. 1, C vs A) or alternate MHC-I processing of HB101.Crl-OVA (Fig. 1, D vs B). These results, consistent with prior data (2, 8, 14), indicate that HB101.Crl-OVA is processed via a post-Golgi, vacuolar mechanism that does not acutely require transit of MHC-I from the ER to the plasma membrane.

Although alternate MHC-I processing of HB101.Crl-OVA did not acutely require transport of nascent MHC-I to the cell surface, the previous experiment did not adequately test the chronic dependence of alternate MHC-I processing or expression of post-Golgi peptide-receptive MHC-I on transport of nascent MHC-I. A reservoir of post-Golgi peptide-receptive MHC-I may persist after short incubations with BFA despite a requirement for replenishment with nascent MHC-I over a longer period. The suggestion that tapasin⁻/⁻ and TAPI⁻/⁻ cells are defective specifically in survival or function of peptide-receptive MHC-I in vacuolar compartments led us to evaluate the relative stability of peptide-receptive MHC-I molecules on tapasin⁻/⁻, TAPI⁻/⁻, and wild-type macrophages. For this purpose, we preincubated macrophages with BFA for various time periods, and SIINFEKL was then added in the continued presence of BFA for 1 h. The macrophages were fixed, and SIINFEKL-Kb complexes were detected with CD80VA1.3 cells. With 60–120 min of exposure to BFA, peptide-receptive MHC-I molecules declined substantially on tapasin⁻/⁻ and TAPI⁻/⁻ macrophages, but were unaltered on wild-type C57BL/6 macrophages (Fig. 2). We conclude that post-Golgi (primarily cell surface) peptide-receptive MHC-I molecules are stably expressed on wild-type cells, but have decreased stability and shorter half-life on tapasin⁻/⁻ and TAPI⁻/⁻ cells. Because of the importance of this observation to vacuolar alternate MHC-I Ag processing, we used additional approaches to test the stability of MHC-I molecules on the cell surface or under conditions reflecting the vacuolar environment (below).

**Temperature shift experiments demonstrate that cell surface Kb stability is decreased on tapasin⁻/⁻ and TAPI⁻/⁻ cells**

Flow cytometry revealed that steady state cell surface expression of Kb is decreased to 32% of the control level on both tapasin⁻/⁻ and TAPI⁻/⁻ macrophages (Fig. 3A). Various groups have suggested that tapasin retains empty or poorly loaded (and therefore unstable) MHC-I molecules in the ER until proper peptide loading.
increasing the half-life and cell surface expression of Kb. To assess incubation with exogenous peptide may stabilize these molecules, including the cell surface) may be poorly loaded and unstable. In- tapasin rrophages, demonstrating decreased stability of peptide-receptive MHC-I on tapasin / and TAP1 / cells. Tapasin /, TAP1 /, and wild-type BMM were incubated with BFA for various time periods before addition of SIINFEKL (in the continued presence of BFA) for 1 h. Macrophages were fixed, washed, and incubated with CD8 OVA1.3 cells as described in Fig. 1. Results are expressed as the mean of triplicate wells ± SD. When error bars are not visible, they are smaller than the symbol width. These results are representative of three independent experiments.

is accomplished (29, 33). In the absence of tapasin, a greater pro- portion of Kb molecules that reach post-Golgi compartments (in- cluding the cell surface) may be poorly loaded and unstable. Incubation with exogenous peptide may stabilize these molecules, increasing the half-life and cell surface expression of Kb. To assess the stability of Kb molecules on tapasin−/−, TAP1−/−, and wild-type cells, we performed temperature shift experiments with and without addition of exogenous peptide (Fig. 3, B and C). Macrophages were incubated overnight at 26°C with or without 5 µM FAPGNYPAL and then shifted to 37°C for various time periods in the presence of 2 µg/ml BFA to prevent egress of nascent MHC-I molecules and to isolate a cell surface cohort of Kb molecules for analysis. Cells were then labeled with AF6-88.5 or CTKb anti-Kb Ab, which both recognize conformation-dependent epitopes on the Kb heavy chain. Kb expression decayed with increasing time at 37°C, indicating the presence of Kb molecules that were unstable at this temperature. This decay was more rapid on tapasin−/− and TAP1−/− macrophages than on wild-type macrophages (Fig. 3, B and C, and Table I). Prior incubation with FAPGNYPAL stabilizing peptide prolonged the half-life of Kb, particularly on tapa- sin−/− and TAP1−/− cells (Fig. 3, B and C, and Table I). Exogenous stabilizing peptide increased the half-life of Kb, as determined with AF6-88.5, from 31 to 166 min on tapasin−/− cells and from 48 to 250 min on TAP1−/− cells (Table I). Similar results were obtained in experiments with CTKb Ab (Table I). The apparent half-life of Kb was calculated as the negative inverse value of the slope of log2(MFV) vs time (see Table I).

Incubation with stabilizing peptides rescues alternate MHC-I processing by tapasin−/− and TAP1−/− macrophages

Incubation with a stabilizing peptide enhances the half-life and partially restores surface expression of Kb on tapasin−/− (Fig. 3) and TAP1−/− cells (Fig. 3) (9, 19). We tested the effect of incubation with stabilizing peptide on subsequent alternate MHC-I Ag processing by tapasin−/− cells compared with TAP1−/− and wild-
type cells. As observed previously with TAP1−/− cells (9), incubation of tapasin−/− cells with stabilizing peptide (FAPGNYPAL or KVVRFDKL) increased alternate MHC-I processing of exogenous particulate Ag (Fig. 4). These results imply that incubation of tapasin−/− and TAP1−/− cells with stabilizing peptide increases peptide-receptive MHC-I molecules that can function in alternate MHC-I processing, apparently via peptide exchange (exchanging stabilizing peptide for antigenic peptide derived from exogenous Ag).

### Kb molecules on tapasin−/− and TAP1−/− macrophages have decreased stability to acidic pH

Tapasin−/− and TAP1−/− cells exhibited a defect in processing of exogenous particulate Ag, but were capable of presenting exogenous peptide (Fig. 1), suggesting that MHC-I molecules in these cells were less able to survive or function in the vacuolar processing environment than on the cell surface. Therefore, we tested the hypothesis that MHC-I molecules expressed by tapasin−/− and TAP1−/− cells were less stable to acidic pH, reflective of the phagolysosomal environment. Macrophages were incubated for 30 min in CBS at various pH values with BFA, washed in DMEM at neutral pH, incubated for 1 h with SIINFEKL in standard medium with BFA, fixed, and assessed for SIINFEKL:Kb complexes with CD8OVA1.3 cells. SIINFEKL binding and presentation by Kb was markedly reduced by incubation at acidic pH (4.5–5.0) with tapasin−/− and TAP1−/− macrophages (Fig. 5, C and E), but not wild-type macrophages (Fig. 5A). These results indicate that peptide-receptive MHC-I molecules on tapasin−/− and TAP1−/− macrophages are more susceptible to destabilization by acidic pH than those on wild-type C57BL/6 cells. This may be due to the relative paucity of high affinity stabilizing peptides bound to MHC-I on the surface of tapasin−/− and TAP1−/− macrophages.

To test the kinetics of acid-induced destabilization of Kb, we incubated macrophages with CBS at pH 5.0 for varying time periods before neutralizing the medium and incubating with SIINFEKL as described above. Presentation of SIINFEKL by tapasin−/− and TAP1−/− macrophages was decreased by preceding acid incubation for 15–30 min, whereas presentation by wild-type macrophages was essentially unaffected by this treatment (Fig. 6). Thus, peptide-receptive MHC-I molecules on tapasin−/− and TAP1−/− macrophages were destabilized by acidic pH within the time frame of their trafficking in phagosomal compartments and function during alternate MHC-I Ag processing, consistent with the hypothesis that the defect in alternate MHC-I processing in tapasin−/− and TAP1−/− cells results from instability of poorly loaded MHC-I to acidic vacuolar pH. In addition, the acid lability of MHC-I on tapasin−/− and TAP1−/− macrophages mirrors the focused deficit of these cells in alternate MHC-I processing of exogenous Ag (Fig. 1). This further suggests that the processing deficit is caused by decreased stability and function of MHC-I molecules in acidic vacuolar processing compartments.

### Acidic pH promotes peptide exchange on MHC-I molecules

Poorly loaded MHC-I molecules are inactivated by acidic pH in the absence of stabilizing peptides, but acidic pH may play a positive role in vacuolar alternate MHC-I processing by promoting peptide exchange, i.e., the binding of antigenic peptide to MHC-I following the acid-catalyzed dissociation of stabilizing peptide from MHC-I. To test this hypothesis, we repeated the previous experiment, but included SIINFEKL both during and after the pH shift incubation (Fig. 5, B, D, and F). Acidic pH actually increased the binding and presentation of SIINFEKL by Kb on wild-type C57BL/6 macrophages (Fig. 5B compared with Fig. 5A), and the presence of SIINFEKL during the acid incubation reversed the acid-induced loss of SIINFEKL binding seen when the peptide was present only after the acid incubation (Fig. 5, D and F, compared with Fig. 5, C and E). These observations indicate that binding of SIINFEKL stabilizes Kb under conditions of acidic pH and an acidic pH environment.
that acidic pH promotes peptide exchange even with K\textsuperscript{b} expressed on wild-type cells that contains high affinity stabilizing peptides.

Incubation at 26°C rescues alternate MHC-I processing by tapasin\textsuperscript{-/-} and TAP1\textsuperscript{-/-} macrophages more than wild-type macrophages, but the presence of peptide at acidic pH rescues or enhances peptide binding. BMM from tapasin\textsuperscript{-/-}, TAP1\textsuperscript{-/-}, or wild-type C57BL/6 mice were incubated for 30 min in CBS with BFA at pH 7.5, 5.5, 5.0, or 4.5 with (B, D, and F) or without (A, C, and E) SIINFEKL. Cells were washed with DMEM, incubated for 1 h at 37°C with SIINFEKL in standard medium with BFA, and fixed. CD8\textsubscript{OVA}1.3 cells were used to determine the presentation of SIINFEKL-K\textsuperscript{b} complexes as described in Fig. 1. Results are expressed as the mean of triplicate wells ± SD. When error bars are not visible, they are smaller than the symbol width. These results are representative of three independent experiments.

Incubation at 26°C rescues alternate MHC-I processing by tapasin\textsuperscript{-/-} and TAP1\textsuperscript{-/-} macrophages and restores acid stability of post-Golgi peptide-receptive MHC-I

Our model indicates that the deficit in alternate MHC-I processing by tapasin\textsuperscript{-/-} and TAP1\textsuperscript{-/-} macrophages stems from decreased stability of the poorly loaded, post-Golgi peptide-receptive MHC-I molecules in the acidic vacuolar environment. To test this hypothesis we stabilized post-Golgi peptide-receptive MHC-I molecules by incubation of cells at 26°C to determine whether this would increase the expression of acid-stable, peptide-receptive MHC-I and thereby enhance alternate MHC-I processing. Incubation of TAP-deficient APCs at subphysiological temperature (26°C) allows stable expression of poorly loaded MHC-I molecules that are unstable at 37°C (35, 37). We demonstrated that incubation of tapasin\textsuperscript{-/-} cells at 26°C similarly increased K\textsuperscript{b} expression over the level seen at 37°C (Fig. 3 and data not shown). Thus, poorly loaded MHC-I molecules on tapasin\textsuperscript{-/-} and TAP1\textsuperscript{-/-} cells are stabilized at 26°C, contributing to a larger population of post-Golgi peptide-receptive MHC-I. Furthermore, incubation at 26°C fully rescued Ag processing by tapasin\textsuperscript{-/-} and TAP1\textsuperscript{-/-} macrophages (Fig. 7). Thus, at 37°C vacuolar alternate MHC-I Ag processing requires TAP and tapasin to generate peptide-receptive MHC-I molecules
of sufficient thermostability. At 26°C, however, tapasin and TAP functions are not necessary, implying that the poorly loaded MHC-I molecules expressed by tapasin−/− and TAP1−/− cells are stable and function well in vacuolar processing at 26°C (this observation also implies that TAP does not transport the peptides derived from exogenous Ags that are cross-presented to CD8 T cells).

If incubation at 26°C restores alternate MHC-I processing function of MHC-I molecules on tapasin−/− and TAP1−/− cells, our model predicts that these molecules should be stable to the acidic vacuolar environment at this temperature. To test this hypothesis, macrophages were incubated for 18 h at 26 or 37°C, incubated at pH 5.0 or 7.5 (with BFA), neutralized, exposed to SIINFEKL (with BFA) for 1 h at 26 or 37°C, and fixed. CD8OVA1.3 cells were used to determine the presentation of SIINFEKL:Kb complexes. Fig. 8 shows that incubation of TAP1−/− and tapasin−/− macrophages at 26°C prevented the destruction of Kb peptide binding capacity at pH 5.0. At 26°C tapasin−/−, TAP1−/−, and wild-type macrophages presented SIINFEKL with similar efficiency after incubation at pH 5.0 or pH 7.5, but at 37°C presentation of SIINFEKL by tapasin−/− and TAP1−/− macrophages was substantially reduced by incubation at pH 5.0. Thus, at 26°C the restoration of alternate MHC-I Ag processing by tapasin−/− and TAP1−/− cells correlates with the restored acid stability of MHC-I, consistent with our model. Together these results suggest that vacuolar alternate MHC-I Ag processing involves binding of peptides to peptide-receptive MHC-I molecules in acidic vacuolar compartments via a peptide exchange mechanism.

**Discussion**

This is the first study to examine alternate MHC-I Ag processing in tapasin−/− cells. In normal cells, tapasin promotes retention of MHC-I molecules in the ER until they are properly loaded with high affinity peptides (33), whereas tapasin−/− cells produce poorly loaded MHC-I molecules that exit the ER. Hypothetically, we considered that a large proportion of post-Golgi MHC-I on tapasin−/− cells might be peptide-receptive, producing enhanced alternate MHC-I Ag processing in tapasin−/− cells. In contrast, our results indicate that alternate MHC-I processing is diminished in both tapasin−/− and TAP1−/− macrophages relative to control macrophages. Although MHC-I traffic to the cell surface is unimpaired in tapasin−/− cells, whereas MHC-I is subject to increased ER retention in TAP1−/− cells, tapasin−/− and TAP1−/− macrophages were similarly deficient for vacuolar MHC-I Ag processing. This appears to result from the instability of peptide-receptive MHC-I on both tapasin−/− and TAP1−/− cells.

Flow cytometry and Ag presentation experiments both indicated that MHC-I molecules on tapasin−/− and TAP1−/− macrophages are substantially less stable than those on wild-type C57BL/6 macrophages. The short half-life of Kb molecules on both TAP1−/− and tapasin−/− cells is apparently caused by loading with low affinity peptides that dissociate rapidly at 37°C. Dissociation of peptide leads to loss of MHC-I heavy chain conformation recognized by the AF6-88.5 and CTKb Abs and/or degradation of MHC-I. Overnight incubation of these cells with FAPGNYPAL peptide increased the apparent half-life of Kb on both tapasin−/− and TAP1−/− macrophages by 5-fold (measured with AF6-88.5) to 10-fold (measured with CTKb).

Prior incubation of tapasin−/− or TAP1−/− macrophages with exogenous stabilizing peptides of high affinity stabilizes MHC-I and enhances alternate MHC-I Ag processing. This observation implies that the processing mechanism includes the exchange of stabilizing peptide for antigenic peptide derived from exogenous Ag. Peptide exchange is possible because components of the MHC-I complex (MHC-I heavy chain, β2-microglobulin, and peptide) bind each other in a reversible and dynamic fashion (37, 45–47). It may seem paradoxical that the binding of high affinity peptides to MHC-I promotes a processing mechanism based on peptide exchange, but enhanced MHC-I survival conferred by stabilizing peptide enables MHC-I to survive for participation in processing, which apparently outweighs considerations of peptide competition. Furthermore, high affinity peptides may stabilize MHC-I molecules to conditions, such as acid pH, that otherwise lead to destruction of MHC-I molecules that are poorly loaded (i.e., occupied with low affinity peptides, which predominate in tapasin−/− and TAP1−/− cells). While binding of peptides with extremely high affinity for MHC-I, e.g., SIINFEKL, may diminish subsequent peptide exchange and alternate MHC-I processing (9), our data indicate that high affinity peptides (e.g., FAPGNYPAL) can serve as stabilizing peptides to promote subsequent binding and presentation of antigenic peptides loaded in vacuolar compartments by peptide exchange in the course of alternate MHC-I Ag processing.

Our model, involving peptide exchange, contrasts with models of some other groups that propose the use of empty MHC-I molecules in alternate MHC-I Ag processing. Schirmbeck and Reimann (48) suggest that alternate MHC-I processing of hepatitis
B surface Ag requires empty Ld molecules. The empty molecules were defined as such by binding of the 64-3-7 Ab shown by Hansen and colleagues to bind Ld molecules not recognized by 30-5-7, which recognizes conformed, peptide-loaded Ld (45, 46, 49–51). Nonetheless, Ld molecules recognized by 64-3-7 could be bound by peptides that fail to invoke the conformation recognized by 30-5-7, but partially stabilize the L complexes. In addition, the nature and affinity of peptide binding required for stabilization may vary with different MHC-I molecules, and the extent of MHC-I stabilization required may vary for different vacuolar processing environments (e.g., the compartments that contribute to processing of hepatitis B surface Ag (48, 52) may be less hostile to MHC-I stability than the phagolysosomal processing environment for HB101.Crl-OVA used in our study). The peptide exchange model for alternate MHC-I Ag processing or presentation of exogenous peptide is also supported by data from several other groups (45, 50, 53, 54).

The vacuolar processing environment may provide conditions, such as acid pH, that destabilize MHC-I complexes and encourage peptide exchange, allowing MHC-I molecules that are occupied with relatively high affinity peptides to undergo peptide exchange and contribute to vacuolar alternate MHC-I presentation. Thus, acidic pH has dual effects in this model, both destabilizing MHC-I peptide exchange, allowing MHC-I molecules that are occupied to MHC-I stability than the phagolysosomal processing environment for HB101.Crl-OVA used in our study). The peptide exchange model for alternate MHC-I Ag processing or presentation of exogenous peptide is also supported by data from several other groups (45, 50, 53, 54).

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