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Bacterial Proteins Can Be Processed by Macrophages in a Transporter Associated with Antigen Processing-Independent, Cysteine Protease-Dependent Manner for Presentation by MHC Class I Molecules

Daniel J. Campbell, Thomas Serwold, and Nilabh Shastri

MHC class I molecules present peptides derived primarily from endogenously synthesized proteins on the cell surface as ligands for CD8+ T cells. However, CD8+ T cell responses to extracellular bacteria, virus-infected, or tumor cells can also be elicited because certain professional APCs can generate peptide/MHC I complexes from exogenous sources. Whether the peptide/MHC-I complexes are generated because the exogenous proteins enter the classical cytosolic, TAP-dependent MHC-I processing pathway or an alternate pathway is controversial. Here we analyze the generation of peptide/MHC-I complexes from recombinant Escherichia coli as an exogenous Ag source that could be delivered to the phagosomes or directly into the cytosol. We show that peritoneal and bone marrow macrophages generate peptide/MHC-I complexes by the classical as well as an alternate, but relatively less efficient, TAP-independent pathway. Using a novel method to detect proteolytic intermediates we show that the generation of the optimal MHC-I binding peptide in the alternate pathway requires cytochrome c as well as other protease(s). This alternate TAP-independent pathway also operates in vivo and provides a potential mechanism for eliciting CD8+ T cell responses to exogenous Ags. The Journal of Immunology, 2000, 164: 168–175.

Major histocompatibility complex class I (MHC-I) molecules bind to short peptides and present them to CD8+ cytotoxic T cells (1, 2). The majority of these peptides are generated through proteolysis of endogenously synthesized cellular proteins in the cytosol (3, 4). Although multiple proteolytic systems have been implicated in this process, the principal activity thought to be responsible for the generation of antigenic peptides is the multicatalytic proteosome present in the cytosol and nucleus of all cells (5). The peptides are subsequently imported into the endoplasmic reticulum by the TAP transporter, where they are loaded onto MHC-I molecules. The trimeric complexes of peptide, MHC class I heavy chain, and β2-microglobulin are then transported to the cell surface via the constitutive secretory pathway for CD8+ T cell recognition. Although this pathway apparently excludes exogenous proteins from presentation by MHC-I molecules, many Ags with no obvious means of accessing the cytosol can nonetheless elicit potent CTL responses in vivo. These include infectious organisms such as intravascular bacteria and parasites (6, 7), viruses and viral products (8–11), cross-primed minor histocompatibility Ags (12–14), and others (15–22).

In vitro experiments have demonstrated that both macrophages (23–26) and dendritic cells (27–30) are capable of processing exogenous protein Ags for presentation by MHC-I molecules. Although presentation of soluble Ags via macrophagocytosis has been demonstrated (23, 29), Ags in particulate or aggregated forms are more efficiently presented by MHC-I in vitro and elicit potent CD8+ CTL responses in vivo (10, 25, 26, 31). Due to their constitutive or inducible expression of B7 costimulatory molecules, dendritic cells and macrophages are both considered professional APCs that are capable of stimulating naive T cells (32). The processing and presentation of exogenous Ags by these APCs could therefore explain the ability of particulate Ags to effectively prime CTL responses in vivo. Elucidating the mechanism by which exogenous Ags are processed is therefore essential to understanding how CTL responses are generated to these Ags.

Two alternative models can explain how APC process phagocytosed material for presentation by MHC-I molecules. In the first model the phagocytosed Ag gains entry into the cytosol and thereby into the classical, cytosolic MHC-I presentation pathway. For instance, phagocytosis of indigestible latex or iron oxide beads targets the associated protein Ags to the cell cytosol through phagocytic overload or cellular indigestion (26, 27, 33, 34). By contrast, protein aggregates, viral particles, or recombinant bacteria are processed for MHC-I presentation in a noncytosolic alternate MHC-I pathway in which Ags are apparently processed within the acidic phagosomes and lysosomes (25, 35–37). However, the relative efficiencies of these pathways and the proteolytic mechanisms for generating the antigenic peptides are not clear.

In this study we used Escherichia coli expressing antigenic fusion proteins to study the mechanisms by which exogenous Ags are processed and presented by MHC-I after bacterial phagocytosis by murine macrophages. By coexpressing a cytoplasmic version of the Listeria monocytogenes hemolysin listeriolysin O (cLLO), we

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1 Abbott Group used in this paper: MHC-I, MHC class I; cLLO, cytoplasmic version of the Listeria monocytogenes hemolysin listeriolysin O; BMDM, bone marrow macrophages; PMö, peritoneal macrophages; CPB, cytoplasmic version of the Listeria monocytogenes OVA257-264; CPY, carboxypeptidase Y; CPRG, chlorophenol red-β-D-galactopiranoside; GFP, green fluorescent protein; MBP, maltose binding protein; LacZ, β-galactosidase; TAP+, mice with a targeted deletion in the TAP1 gene; IPTG, isopropyl β-D-thiogalactoside.

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3 Abbreviations used in this paper: MHC-I, MHC class I; cLLO, cytoplasmic version of the Listeria monocytogenes hemolysin listeriolysin O; BMDM, bone marrow macrophages; PMö, peritoneal macrophages; CPB, cytoplasmic version of the Listeria monocytogenes OVA257-264; CPY, carboxypeptidase Y; CPRG, chlorophenol red-β-D-galactopiranoside; GFP, green fluorescent protein; MBP, maltose binding protein; LacZ, β-galactosidase; TAP+, mice with a targeted deletion in the TAP1 gene; IPTG, isopropyl β-D-thiogalactoside.
also targeted the same fusion protein to the classical, cytotoxic MHC-I processing pathway, thereby allowing a direct comparison of both the efficiency and processing mechanisms used to present cytotoxic and exogenous Ags. We find that both murine bone marrow and peritoneal macrophages can present peptide/MHC-I complexes derived from Ags expressed in E. coli, and coexpression of cLLO enhanced this presentation by 1000-fold. The presentation of the exogenous Ag in the absence of cLLO was not due to leakage from the phagosomes to the cytosol, as it was independent of functional TAP. By contrast, inhibiting cysteine proteases blocked both Ag presentation and the recovery of antigenic peptides in cell extracts. Using an enzymatic system to detect proteolytic intermediates in the processing of these exogenous Ags, we found that noncysteine proteases also participate in the breakdown of these Ags, but fail to produce the optimal antigenic peptide and other small peptides that are the likely precursors of the antigenic peptide. Finally, by recovering cells from mice infected with antigenic E. coli, we demonstrate that this alternate pathway is used by macrophages in vivo and thus provides a potential mechanism for the initiation of CD8+ T cell responses to bacterial Ags.

Materials and Methods

Cells and mice

All cells were maintained in RPMI 1640 supplemented with 10% FBS, 2 mM glutamine, 1 mM pyruvate, 50 mM 2-ME, 100 U/ml penicillin, and 100 µg/ml streptomycin (complete RPMI) at 37°C in a 5% CO₂, air atmosphere. The LacZ-inducible, OVA-specific T cell hybridoma B3Z has been previously described (38). The F1/SR.Z T cell hybrid recognizes a minor histocompatibility Ag expressed by B6 cells (39). The dendritic cell line DC2.4 was a gift from Dr. K. Rock (University of Massachusetts, Boston, MA) (27). Male and female C57BL/6 and B6CBAF1/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used between the ages of 2–12 mo. B6.129-Tap1tm1Arp (TAP1 chased from The Jackson Laboratory (Bar Harbor, ME) and used between

E. coli strains and constructs

All recombinant E. coli were derived from the Top10F’ K12 strain (Invitrogen, San Diego, CA). The MBP-OVA construct was prepared by subcloning the PstI/XhoI fragment of the OVA cDNA into the PstI/XhoI sites of the pEYRF vector (40) and then ligating the BamHI/XhoI fragment of this construct into the pMal-P2 vector (New England Biolabs, Beverly, MA). The mutated MBP-OVA construct, used to detect Ag fragments with trypsin/carboxypeptidase B (CPB) digestion, was made by the same method using an OVA cDNA that contained the mutations indicated in Fig. 1. The cLLO construct made in the pACYC184 vector has been described previously (41) and was a gift from Drs. Darren Higgins and Dan Portnoy (Department of Molecular and Cell Biology, University of California). The pTrc-GFP construct was made by subcloning the GFP coding sequence on an Ncol/EcoRI fragment from the MSCV2.2 vector into unique Ncol/EcoRI sites in the E. coli expression vector pTrcHisC. In all Ag presentation experiments, confluent overnight cultures of MBP-OVA E. coli were diluted 1/10 in LB or LBpromisc for Ag+ cLLO strains and were grown with shaking at 37°C for 45 min before Ag expression was induced by addition of 1 mM isopropyl β-D-thiogalactoside (IPTG). Bacteria were incubated for another 4 h at 37°C before being used as an Ag source in all experiments. For cell recovery experiments, bacteria expressing GFP and MBP-OVA were handled identically, except no IPTG was added during culture.

T cell stimulation assays

Bacteria or peptides were added to BM-Mφ or peritoneal Mφ (PMφ) (105/well) in antibiotic-free RPMI in 96-well plates as indicated. The cultures were spun for 2 min at 850 × g before being incubated at 37°C for 1 h to allow phagocytosis. The cells were then washed twice with 200 µl of PBS/well to remove extracellular bacteria or unbound peptides, and B3Z T cells (105/well) were added to complete RPMI and the IFN-γ assay. The HPLC fractions or cell extracts were treated with trypsin/CPB as indicated and diluted in RPMI before adding DC2.4 (5 × 105/well) as APC and B3Z T cells (105/well). In experiments using peritoneal APC recovered from infected mice, the recovered cells were titrated in 96-well plates as indicated before adding either B3Z or F1/SR.Z T cell hybrids (105/well) in complete RPMI and 100 µg/ml gentamicin. In all experiments, T cell activation was assessed after 72 h of culture using 100 µg/ml gentamicin. Material retained on the filter was recovered by vigorous pipetting with 500 µl of 10% acetic acid. The filtrate and retained bacteria were then dried and resuspended in 200 µl of PBS. The material was then fractionated on an HPLC pump using a 2.2-µm 250-mm C18 column with 5-% acetonitrile in water (buffer A) and 0.1% trifluoroacetic acid in acetonitrile (buffer B). The peptides were separated by HPLC analysis.

HPLC analysis

Cellular extracts were fractionated on a Hewlett Packard 1050 quaternary pump HPLC using a 2.2- × 250-mm C18 column with 5-% particle size and 300-nm pores (Vydac, Hesperia, CA). Samples were separated using an acetonitrile/water gradient with trifluoroacetic acid as the ion-pairing agent. Solvents used were 0.1% trifluoroacetic acid in water (buffer A) and 0.1% trifluoroacetic acid in acetonitrile (buffer B). The peptides were separated using a 25-min gradient starting at 23% B and ending at 48% B. Five-drop fractions were collected in 96-well plates. The fractions were then dried in a vacuum centrifuge, treated with trypsin/CPB, and tested for B3Z stimulating peptides with or without trypsin/CPB treatment as indicated. For Mφ extracts, adherent bone marrow cells obtained as described above were cultured overnight in six-well plates (3 × 106/well) in RPMI and 100 U/ml murine IFN-γ. The cells were washed once with PBS and overlaid with antibiotic-free RPMI with or without the cysteine protease inhibitor mixture containing E64 (50 µM; Sigma), leupeptin (0.1 mM; Sigma), and ZFA-FMK (25 µM; Enzyme Systems, Livermore, CA). The cells were cultured at 37°C for 30 min before 7.5 × 105 bacteria were added to each well. The cells were spun for 2 min at 850 × g and cultured for 1 h at 37°C to allow phagocytosis. The cultures were then harvested and washed three times with 10 ml of PBS, spinning the cells for 4 min at 200 × g in between each wash to remove any remaining extracellular bacteria. Pelleted cells were then extracted in 500 µl of 10% acetic acid and 2 µM of an irrelevant peptide and boiled for 10 min. Cell debris was then pelleted, and the supernatant was passed through a 10-kDa cut-off filter (Millicore, Bedford, MA). Material retained on the filter was recovered by vigorous pipetting with 500 µl of 10% acetic acid. The filtrate and retained bacteria were then dried and resuspended in 200 µl of PBS. The material was then tested for B3Z stimulating peptides with or without trypsin/CPB treatment as described below.

Trypsin/CPB treatment

One hundred microliters of Mφ or bacterial extract was digested by adding 10 µl of 2 mg/ml trypsin and 1 µl of 5 mg/ml CPB. Dried HPLC fractions were resuspended in 30 µl of PBS containing 0.2 mg/ml trypsin and 50 µg/ml CPB and incubated for 4 h at 37°C before being used in the B3Z T cell stimulation assay described above.

Inhibitor assays

The BM-Mφ (105/well) were cultured in 96-well plates overnight in RPMI and IFN-γ as described above. After washing the adherent cells with 200 µl of PBS, the cells were incubated with antibiotic-free RPMI containing pepstatin A (1 µM; Sigma), E64 (50 µM), leupeptin (0.1 mM), or ZFA-FMK (25 µM) and cultured at 37°C for 30 min before addition of bacteria or the SHL8 peptide. The cells were spun for 2 min at 850 × g and cultured for 1 h at 37°C. After two washes with 200 µl of PBS/well the B3Z T cells were added (105/well) in RPMI, 100 µg/ml gentamicin, and the indicated
inhibitor. T cell activation was measured 5 h later with the CPRG substrate as described above.

In vivo phagocytosis

C57BL/6 or TAP1−/− mice were injected i.p. with 5 × 10⁷ E. coli expressing MBP-OVA, GFP, or His-UTY, prepared as described above. Peritoneal cells were recovered 2 h later by peritoneal lavage with 4–5 ml of PBS. Contaminating RBC were removed as necessary by resuspending the pelleted cells in 0.5 ml of water and immediately diluting with 15 ml of PBS. The cells were then washed twice with 10–15 ml of PBS to remove any extracellular bacteria and used in T cell stimulation assays or stained and analyzed by flow cytometry as follows. Abs used were 16–10A1 (α-B7.1), GL1 (α-B7.2), M1/70 (α-CD11b), N418 (α-CD11c), and RB6–8C5 (α-GR-1). The Fc receptors were first blocked by incubating cells in 50 μl of mouse serum for 20 min. Primary Abs were added as 1/100 dilutions of ascites or purified Ab in PBS/2% FCS or as undiluted culture supernatants and incubated for 20 min. Appropriate PE-conjugated secondary Abs (Caltag, Burlingame, CA) were then added as necessary, and cells were incubated for 20 min. Samples were analyzed with either a Coulter XL (Hialeah, FL) or Becton Dickinson FACS Caliber (Mountain View, CA) flow cytometer.

Results and Discussion

Processing and presentation of exogenous recombinant E. coli as peptide/MHC-I complexes in macrophages

Recombinant E. coli expressing antigenic precursor proteins were prepared to study the processing mechanisms for generation of peptide/MHC-I complexes from exogenous Ags. A fusion protein was constructed with the maltose binding protein and residues 138–386 of OVA (MBP-OVA), which contains the SIINFEKL (SL8) octapeptide. The SL8 peptide is presented by Kb MHC-I molecule on the cell surface and is recognized by the LacZ-inducible, B3Z T cell hybridoma (38). To allow the detection of not only the final processed SL8/Kb complex by T cell activation but also potential proteolytic intermediates, the sequences of the SL8 peptide and its flanking residues were modified. The lysine (K) at the seventh position of SL8 peptide was changed to a histidine (H) residue, and the N- and C-terminal flanking residues were changed to lysines. These substitutions enable the proteases trypsin, which cleaves at the carboxyl terminus of lysine residues, and CPB, which removes a single carboxyl-terminal lysine residue, to liberate the optimally active SHL8 peptide from poorly active large polypeptide fragments (43). This strategy allows for the sensitive detection of large SHL8-containing fragments in cellular and bacterial extracts via T cell activation assays (Fig. 1A).

We first ascertained the extent to which this antigenic fusion protein was degraded in the recombinant E. coli itself. Bacterial extracts were prepared by repeated freeze-thawing, acid extracted with 10% acetic acid, and passed through a 10-kDa cut-off filter. As expected for the ~70-kDa MBP-OVA protein, T cell-stimulating activity was recovered in the >10-kDa retentate and the <10-kDa filtrate were then assayed for B3Z stimulating activity without any treatment or after digestion with trypsin and CPB. As expected for the ~70-kDa MBP-OVA protein, cell-stimulating activity was recovered in the >10-kDa retentate and was enhanced by about 200-fold after treatment with trypsin/CPB (Fig. 1B). Trypsin/CPB treatment had no effect on the activity of synthetic SHL8 peptide, demonstrating that the observed enhancement is not due to any nonspecific effects of the enzymes on the APC or the T cells (data not shown). In contrast to the retentate, T cell-stimulating activity was not detected in the <10-kDa filtrate, even after trypsin/CPB treatment (Fig. 1C). Thus, as expected the ~70-kDa MBP-OVA protein and its potential >10-kDa proteolytic fragments were retained by the >10-kDa cut-off filter, and digestion of large protein fragments with trypsin/CPB released the optimally active SL8 peptide. Most importantly, even if some MBP-OVA precursor was degraded in the E. coli, these fragments were >10 kDa and would therefore require proteolytic processing in the APC to generate the SL8/Kb complex.

Next, murine macrophages were used as APC for generating the SHL8/Kb complex after coculture with E. coli expressing MBP-OVA. Both PMφ and BMMφ generated the peptide/Kb complex when fed MBP-OVA E. coli in a dose-dependent manner (Fig. 2A). This stimulation was Ag specific, as no B3Z response was detected when Mφ were cocultured with E. coli that expressed other antigenic precursors or were fixed before culture with the bacteria (data not shown). Identical results were obtained using E. coli expressing either an unmodified MBP-OVA fusion or a histidine-tagged OVA fusion protein, demonstrating that presentation of this exogenous Ag was not dependent on either the sequence changes made to the antigenic peptide and its flanking amino acids or to the MBP portion of the protein (data not shown). To compare the processing of these exogenous Ags with those that can directly access the endogenous, cytosolic MHC-I pathway, we also used E. coli coexpressing the same MBP-OVA antigenic precursor along with cLLO. Like the wild-type LLO, cLLO can also disrupt the phagosomal membrane but because cLLO is not secreted, it requires prior digestion of bacteria in the phagosomes. The bacterial proteins then gain access to the host cell cytosol where they enter the classical MHC-I processing pathway (41). Again, both PMφ and BMMφ presented the SHL8/Kb complexes after phagocytosis of MBP-OVA- and cLLO-expressing E. coli (Fig. 2B). However, targeting the MBP-OVA protein to the cytosol by the coexpression of...
of cLLO enhanced the presentation of the SHL8/Kb complex by about 1000-fold. This enhancement could be attributed to the altered localization of the antigenic precursor and not to the expression level of MBP-OVA, because Western blot analysis with anti-OVA Abs showed that equivalent amounts of the protein were produced by E. coli regardless of whether cLLO was coexpressed in the cells (Fig. 2C). We conclude that macrophages can generate peptide/MHC-I complexes using exogenous Ags and that the efficiency of presentation correlated with the intracellular localization of the Ag.

The MHC-I presentation of E. coli expressing MBP-OVA is TAP1 independent

The MBP-OVA expressed in E. coli was processed and presented by MHC-I without obvious access to the host cell cytosol and the conventional MHC-I processing pathway. It was however, possible that a small amount of the antigenic protein had leaked from the phagosomes into the cytosol through phagocytic overload or “cellular indigestion” (26, 27, 33). Alternatively, the antigenic protein could be processed and loaded on to MHC-I in an alternate MHC-I pathway that did not require access to the cytosol (25, 36). To distinguish between these possibilities, we used macrophages from mice with a targeted deletion in the TAP1 gene (TAPo mice). Cells from TAP0 mice cannot import antigenic peptides produced within the cytosol into the endoplasmic reticulum where they are loaded onto MHC-I molecules and as a consequence are severely compromised in their presentation of endogenous peptide/MHC-I complexes (44). Notably, the presentation of SHL8/Kb complexes derived from the exogenous E. coli expressing MBP-OVA alone as well as the synthetic SHL8 peptide was completely TAP1 independent (Fig. 3. A and C). In contrast, only the cells from wild-type mice, but not those from TAP0 mice, stimulated the B3Z cells when cultured with E. coli expressing MBP-OVA and cLLO (Fig. 3B). These results show that both the classical cytosolic, TAP-dependent and an alternate TAP-independent pathway can be used to generate peptide/MHC-I complexes from exogenous Ags. These findings are in agreement with earlier descriptions of a TAP-independent presentation pathway from other laboratories (25, 36, 45, 46). In comparing responses to the same antigenic precursor with or without cLLO, our results further establish that the TAP-dependent cytotoxic pathway is about 3 orders of magnitude more efficient than the TAP-independent pathway.

The processing of MBP-OVA to SHL8 requires cysteine proteases

The principal cytosolic proteolytic activity implicated in generating MHC-I peptides is the multicatalytic proteosome (4, 5). However, MBP-OVA introduced exogenously into macrophages within E. coli does not require cytosolic access for MHC-I presentation, and proteasomes have not been reported within the acidic compartments where these Ags are targeted. Therefore, distinct proteolytic activities must mediate the generation of antigenic peptides in the alternate MHC-I processing pathway used by these cells. Acid proteases, cathepsins D and E, and cysteine proteases, cathepsins B, H, L, and S, are found extensively in acidic compartments and have been implicated in the proteolytic processing of exogenous Ags for presentation by MHC-II molecules (47–50) as well as the MHC-II-associated invariant chain (51, 52). Using chemical inhibitors, we addressed the roles of these two classes of proteases in the generation of antigenic peptide/MHC complexes from MBP-OVA in macrophages. Pepstatin A, an acid protease inhibitor, had little effect on the presentation of MBP-OVA. However, the cysteine protease inhibitors E64, leupeptin, and ZFA-FMK all effectively blocked B3Z stimulation (Fig. 4A). None of the inhibitors tested had any effect on B3Z response to synthetic SHL8 peptide, demonstrating that these chemicals were not generally inhibiting the ability of Mφ to present or T cells to respond to MHC-I Ags (Fig. 4B).

To define the proteolytic event(s) that was blocked during the generation of the SL8/Kb complex by the cysteine protease inhibitors, we cocultured BMMφ with E. coli expressing MBP-OVA and extracted the antigenic peptide pool generated in untreated cells or cells treated with a mixture of cysteine protease inhibitors (CPI = E64 + leupeptin + ZFA-FMK). The extracts were passed through a 10-kDa cut-off filter, and the filtrates were assayed for the presence of B3Z stimulating peptides as such or after digestion with trypsin and CPB to release the SHL8 peptide from larger
inactive peptides. The SHL8 antigenic activity was readily detected in the <10-kDa filtrate of untreated cells, but was undetectable in the extract from cells treated with the cysteine protease inhibitors (Fig. 4C). Interestingly, treatment of the filtered extracts with trypsin/CPB revealed that SHL8-containing, smaller than 10-kDa antigenic fragments were nevertheless generated in both untreated and cysteine protease inhibitor-treated macrophages (Fig. 4D), indicating that their generation is at least partially cysteine protease independent. Because these <10-kDa fragments were undetectable in the E. coli before coculture with APC (Fig. 1C), their presence in the cell extracts indicates that they were generated during the processing of bacterial Ags in the macrophages. Note that the formal possibility that some proteolysis may have occurred due to bacterial proteases that were up-regulated after phagocytosis into the macrophages has not been ruled out.

To further elucidate the cysteine protease-dependent and independent proteolytic steps, the <10-kDa filtrates were fractionated by reverse phase HPLC, and each fraction was assayed for B3Z stimulating peptides after trypsin/CPB treatment. The HPLC profiles showed that both quantitative and qualitative changes had occurred in cells treated with cysteine protease inhibitors on the generation of SHL8-containing antigenic fragments (Fig. 5). In addition to the SHL8 peptide (arrowhead), which is the final product presented by the K\textsuperscript{b} molecule (53, 54), at least five other SHL8-containing peptides were detected in the extracts of untreated cells. These peaks span the entire range of the acetonitrile gradient used to separate them, indicating that peptides of various sizes and hydrophobicities are generated. By contrast, in the extract of cells treated with the cysteine protease inhibitors, a single peak of activity was recovered in fractions 54–57. The late elution time of this peak on a reverse phase C18 column suggests that it represents a larger hydrophobic peptide(s). Recovery of each of the smaller, relatively hydrophilic antigenic peptides, including the optimal SHL8 peptide, was completely ablated by these inhibitors. This analysis directly demonstrates that cysteine proteases are required for proteolysis of exogenous Ags to the smaller peptides, which are likely to be the immediate precursors of the optimal peptide. Furthermore, the relatively minor inhibitory effect on the generation of the larger SHL8-containing peptide(s) suggests that other proteases, resistant to the panel of inhibitors tested here, are likely to be involved in the alternate Ag processing pathway. We conclude that processing of exogenous MBP-OVA in the noncytosolic pathway generates several proteolytic intermediates as well as the optimal peptide and that this process requires cysteine and possibly other proteases.

**FIGURE 5.** Cysteine protease inhibitors block the generation of SHL8 and other proteolytic products in Mφ cultured with E. coli expressing MBP-OVA. Extracts of bone marrow Mφ cultured with E. coli expressing MBP-OVA in the presence (●) or the absence (○) of the cysteine protease inhibitor (CPI) mixture were prepared as described in Fig. 4. Extracts were fractionated by reverse phase HPLC, and each fraction was treated with trypsin/CPB and tested for B3Z stimulating peptides using DC2.4 cells as APC. ○, Response of T cells to fractionated extracts of bone marrow Mφ cultured with E. coli expressing an irrelevant protein. The vertical arrow indicates the peak fractions where synthetic SHL8 peptide elutes under identical conditions.
TAP0 mice were injected with either presentation assays without further purification. B6 wild-type and bacteria, this infection protocol was used to isolate the cells for Ag had phagocytosed the recombinant bacteria.

as well as cells recovered from TAP1 o mice infected with MBP-OVA (Fig. 6 B7.1 (B), B7.2 (C), CD11c (D), GR-1 (E), or CD11b (F). The Abs used in B and C were directly conjugated to PE, while cells in samples A, D, E, and F were subsequently incubated with appropriate PE-conjugated secondary Abs. All samples were analyzed by two-color flow cytometry.

FIGURE 6. Presentation of bacterial Ags by APC recovered from infected mice. A. B6 mice were injected i.p. with $5 \times 10^7$ E. coli expressing MBP-OVA (left panel) or GFP (right panel). Cells were harvested 2 h later by peritoneal lavage with PBS and analyzed by flow cytometry for GFP fluorescence. B. Left panel. Cells recovered from wild-type mice infected with MBP-OVA (●) or the control (○) irrelevant His-Uty expressing E. coli as well as cells recovered from TAPI* mice infected with MBP-OVA E. coli (○) were titrated as indicated and tested for their ability to stimulate B3Z T cells. Right panel. Cells recovered from wild-type (●) or TAPI* (○) mice infected with MBP-OVA E. coli were titrated as indicated and tested for their ability to stimulate the minor histocompatibility Ag-specific F1/5R.SZ T cells.

expressing GFP. Two hours later, cells were harvested from these mice by peritoneal lavage and analyzed by flow cytometry. A large fraction (~70%) of the cells recovered from the peritoneal cavity were GFP+ (Fig. 6A, right) compared with cells recovered from mice injected with nonfluorescent bacteria expressing MBP-OVA (Fig. 6A, left), indicating that a large fraction of peritoneal cells that had phagocytosed the recombinant bacteria.

Because a majority of the recovered cells had phagocytosed bacteria, this infection protocol was used to isolate the cells for Ag presentation assays without further purification. B6 wild-type and TAP0 mice were injected with either E. coli expressing MBP-OVA or the irrelevant His-Uty protein as a nonantigenic control. The recovered cells were tested for their ability to stimulate B3Z T cells. Cells recovered from both wild-type and TAP0 mice infected with MBP-OVA E. coli stimulated B3Z cells, while cells recovered from mice infected with His-Uty expressing bacteria did not (Fig. 6B, left). We therefore conclude that these cells not only present bacterial proteins on MHC-I, but also that they can use a TAP-independent processing pathway to do so. As a control for presentation of endogenously synthesized Ags, the cells recovered from wild-type and TAP0 mice were also tested for their ability to stimulate F1/5R.SZ T cells, which are specific for a minor histocompatibility Ag expressed by B6 mice. The stimulation of F1/5R.SZ was completely ablated in TAP0 cells, establishing that the TAP0 cells that presented exogenous bacterial Ags were incapable of presenting endogenously processed Ags (Fig. 6B, right).

The ability to label the phagocytic APC with bacteria expressing GFP allowed analysis of the surface phenotype of these cells. Cells recovered from mice infected with E. coli expressing GFP were further stained with lineage-specific Abs and analyzed by two-color flow cytometry. The CD11b integrin, also known as MAC1, is present on Mφ and neutrophils (55) and was expressed at high levels on all of the GFP+ cells recovered from the peritoneum (Fig. 7, A and F). By contrast, the CD11c integrin, which is primarily expressed by dendritic cells (56), was undetectable on these cells (Fig. 7D). Among the CD11b+ cells, we were able to distinguish between neutrophils and Mφ by analyzing the expression of the neutrophil specific GR-1 Ag. GR-1+ neutrophils were about 30% of the GFP+ cells (Fig. 7E), and the remaining 70% were most likely resident PMφ. The expression of the costimulatory ligands B7.1 and B7.2 was also analyzed on these cells. Although B7.1 expression was undetectable (Fig. 7B), a large fraction of the GFP+ cells expressed the potent costimulatory ligand B7.2, and expression of B7.2 correlated with higher levels of phagocytosis as indicated by the high GFP levels in these cells (Fig. 7C). The expression of B7.2 on these cells suggests that they could prime CD8+ T cell responses directed against Ags expressed by invading, noncytotoxic bacteria (32).

Taken together, these results indicate that multiple MHC-I presentation pathways exist for exogenous Ags. This raises the question of why APC need both the cytosolic, TAP-dependent and the noncytotoxic, TAP-independent processing mechanisms. One reason may be that many pathogens, particularly viruses, produce proteins that inhibit the classical MHC-I processing pathway at virtually every step, including Ag proteolysis, TAP transport, peptide loading, and MHC-I transit (57). Because the alternate MHC-I presentation pathway does not depend upon the same proteolytic steps or TAP transport, it may help in eliciting CTL responses
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