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# Analysis of the Role of Bleomycin Hydrolase in Antigen Presentation and the Generation of CD8 T Cell Responses<sup>1</sup>

Charles F. Towne,\* Ian A. York,<sup>†</sup> Levi B. Watkin,\* John S. Lazo,<sup>‡</sup> and Kenneth L. Rock<sup>2\*</sup>

Long oligopeptides (>10 residues) are generated during the catabolism of cellular proteins in the cytosol. To be presented to T cells, such peptides must be trimmed by aminopeptidases to the proper size (typically 8–10 residues) to stably bind to MHC class I molecules. Aminopeptidases also destroy epitopes by trimming them to even shorter lengths. Bleomycin hydrolase (BH) is a cytosolic aminopeptidase that has been suggested to play a key role in generating MHC class I-presented peptides. We show that BH-deficient cells from mice are unimpaired in their ability to present epitopes from N-extended precursors or whole Ags and express normal levels of MHC class I molecules. Similarly, BH-deficient mice develop normal CD8<sup>+</sup> T cell responses to eight epitopes from three different viruses in vivo. Therefore, BH by itself is not essential for the generation or destruction of MHC class I peptides. In contrast, when BH<sup>-/-</sup> mice are crossed to mice lacking another cytosolic aminopeptidase, leucine aminopeptidase, the resulting BH<sup>-/-</sup>leucine aminopeptidase<sup>-/-</sup> progeny show a selective increase in CD8<sup>+</sup> T cell responses to the gp276 epitope from lymphocytic choriomeningitis virus, whereas the ability to present and respond to several other epitopes is unchanged. Therefore, BH does influence presentation of some Ags, although its role is largely redundant with other aminopeptidases. *The Journal of Immunology*, 2007, 178: 6923–6930.

Most nucleated cells express MHC class I molecules on their surface, where they present peptides ranging from 8 to ~11 aa in length. These peptides are derived from intracellular proteins that have been degraded by proteasomes (1, 2). This process allows CTL to monitor cells and eliminate ones producing foreign proteins, e.g., from viruses or mutations.

Most peptides generated by the proteasome are too short (<8 aa) to serve as MHC class I-presented peptides (3), but ~10% of proteasomal products are the proper length to bind to MHC class I molecules (3). Another ~15% of proteasomal products are longer than 10 residues (3, 4) and require further trimming to be presented on class I molecules. The proteasome is the only protease in cells that can generate the proper C terminus of most MHC class I-presented peptides (or their precursors) (5, 6). However, we and others have shown that peptides with as many as 25 extra amino acids at the N terminus can be trimmed by aminopeptidases localized in the cytosol (5–7) and the endoplasmic reticulum (ER)<sup>3</sup> (5, 8–10) to generate mature epitopes.

The major aminopeptidase in mice that trims peptides in the ER for presentation on MHC class I molecules is ER aminopeptidase 1 (ERAP1) (11–13). This metalloprotease rapidly trims N-extended peptides to 9 or 8 mers and then slows or stops (12–14). In many cases, this generates the final mature epitope, while in other cases it destroys the peptide. In the absence of ERAP1, antigenic precursors can still be trimmed in the cytosol, before their transport into the ER (13, 15–17).

The specific aminopeptidases that are involved in trimming antigenic peptides in the cytosol have not been elucidated. Cytosolic aminopeptidases such as tripeptidyl peptidase II, leucine aminopeptidase (LAP), bleomycin hydrolase (BH), and puromycin-sensitive aminopeptidase have all been suggested to play a role in MHC class I peptide generation. However, most of the evidence supporting these suggestions has come from cell-free biochemical studies and such experiments do not prove a role in *in vivo* situations. We and others have recently shown that although LAP trims peptide precursors *in vitro* (18) and in living cells (19, 20), it is not essential for MHC class I peptides in cells or in mice (21).

BH (EC 3.4.22.40) is a conserved cysteine protease that is broadly expressed in human tissues (22). It was named for its ability to hydrolyze the antitumor agent bleomycin and is up-regulated in a number of bleomycin-resistant tumors and cell lines (22–24). However, the physiological role for BH is unknown. It has been suggested that a genetic polymorphism in BH is linked to susceptibility to Alzheimer's disease in humans (25–28), although this is controversial (29–31).

BH has also been suggested to play a role in the generation of MHC class I-presented peptides such as the vesicular stomatitis virus (VSV) nucleoprotein (NP) epitope RGYVYQGL (NP52–59). Purified BH and BH-containing fractions from soluble cell extracts trimmed N-extended precursors of the VSV-NP epitope (7). Treating the cell extracts with the aminopeptidase inhibitor Ala-Ala-Phe-CMK (AAF-CMK) prevented this trimming and treating living cells with AAF-CMK blocked the presentation of this VSV-NP epitope to CTLs (7). Presentation of the immunodominant OVA epitope SIINFEKL from peptide precursors by live cells was also inhibited after treatment with AAF-CMK. Together,

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<sup>3</sup> Abbreviations used in this paper: ER, endoplasmic reticulum; ERAP1, ER aminopeptidase 1; LAP, leucine aminopeptidase; BH, bleomycin hydrolase; VSV, vesicular stomatitis virus; NP, nucleoprotein; MEF, mouse embryonic fibroblast; LCMV, lymphocytic choriomeningitis virus.

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these results indicate that the presentation of multiple epitopes may depend on AAF-CMK-sensitive enzymes, such as BH. Similarly, the cysteine protease inhibitor E-64 was shown to block presentation of one murine CMV epitope (YPHFMPNTL) from a precursor expressed in a recombinant vaccinia minigene (32). However, although E-64 inhibits BH, it also blocks many other proteases (33) and it did not inhibit the generation of the NP52–59 epitope in a different cell line (7). In addition, AAF-CMK also inhibits other aminopeptidases (34–37) and may even inhibit some proteasomal activities (38) as well as other cellular functions.

BH-deficient mice have been previously described (39), but their ability to generate MHC class I-presented peptides or mount proper immune responses has not been examined. To determine the contribution of BH to Ag presentation, we have examined MHC class I Ag presentation in BH-deficient mice and BH-deficient cell lines. In addition, because aminopeptidases may be functionally redundant, we analyzed Ag presentation in mice lacking both BH and LAP.

## Materials and Methods

### Mice and mouse embryonic fibroblasts (MEFs)

BH-deficient mice were initially described by Schwartz et al. (39). BH/LAP-deficient mice were generated by breeding LAP-deficient and BH-deficient mice. C57BL/6 (Thy1.2<sup>+</sup>) and congenic control mice, B6.PL-Thy1a/CyJ (Thy1.1<sup>+</sup>), were purchased from The Jackson Laboratory and were maintained under specific pathogen-free conditions within the Department of Animal Medicine (University of Massachusetts Medical School, Worcester, MA).

MEFs were generated from 12- to 14-day embryos from BH-heterozygote mice (for BH-deficient and wild-type control MEFs), or BH/LAP-deficient mice (for BH/LAP-deficient MEFs), or C57BL/6 mice (for wild-type control MEFs).

### Plasmids, primers, and PCR

A three-primer PCR protocol was used to screen for BH-deficient mice, as described (39). A three-primer PCR protocol was also used to screen for LAP-deficient animals as previously described (21).

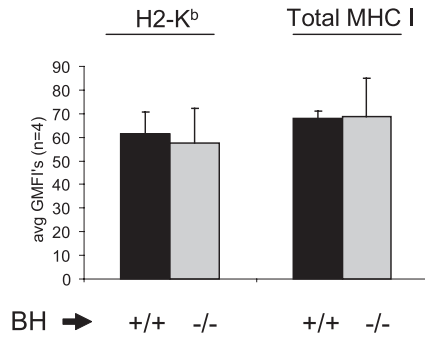
To express N-extended SIINFEKL precursors, we constructed plasmids consisting of ubiquitin with SIINFEKL precursors fused to the C terminus, as previously described (40). C-terminal ubiquitin hydrolases efficiently release peptides from the ubiquitin moiety (41). An internal ribosome entry site downstream of the ubiquitin-SIINFEKL fusion was followed by GFP, used for determining comparable levels of plasmid expression in cells.

### Virus infection of mice

Mice were injected i.p. with  $5 \times 10^4$  PFU/mouse of lymphocytic choriomeningitis virus (LCMV) Armstrong (a gift from Dr. R. Welsh, University of Massachusetts Medical School, Worcester, MA), or with  $5 \times 10^6$  PFU/mouse of recombinant vaccinia containing chicken OVA (Vac-OVA) (42) (provided by Drs. J. Yewdell and J. Bennink, National Institutes of Health, Bethesda, MD). Mice were infected i.v. with  $5 \times 10^6$  PFU/mouse of VSV (a gift from Dr. R. Welsh, University of Massachusetts Medical School). Eight days (LCMV) or 7 days (Vac-OVA and VSV) later, splenocytes were harvested and incubated for 5 h with the appropriate peptide (5  $\mu$ M for LCMV, Vac-OVA, 2  $\mu$ M for VSV), or with anti-CD3 $\epsilon$  (BD Biosciences), all in the presence of GolgiPlug (BD Biosciences) and rIL-2 (BD Biosciences). Peptides that were used to stimulate IFN- $\gamma$  production after vaccinia infection were B8R (TSYKFESV) (43), SIINFEKL, and P10 (STLNFNLL) (44). LCMV peptides included gp33 (KAVYNFATC), gp276 (SGVENPGGYCL), NP205 (YTVKYPNL), and NP396 (FQPQNGQFI) (45, 46). For VSV, the NP52–59 peptide (RGYVYQGL) was used (47). All peptides were synthesized (Anaspec). Cells were stained for CD8, CD44, and intracellular IFN- $\gamma$  using commercial Abs (BD Biosciences), and analyzed by flow cytometry as previously described (21).

### Abs and flow cytometry

The mAb 25.D1.16 (anti-H-2K<sup>b</sup> plus SIINFEKL) (48), AF6-88.5 (anti-H-2K<sup>b</sup>) (49), Y3 (anti-H-2K<sup>b</sup>) (50), M1/42 (anti-H-2K, D, and L) (51), or H36.4.5 (anti-influenza HA) (a gift of W. Gerhard, The Wistar Institute, University of Pennsylvania, Philadelphia, PA) were used as primary Abs in staining MEFs for flow cytometry. After incubation in one of the primary Abs, the cells were washed with PBS and stained with donkey anti-mouse



**FIGURE 1.** MHC class I presentation on MEFs. Wild-type and BH-deficient MEFs were stained with B8.24.8S (H2-K<sup>b</sup>) or with M142 (all MHC class I) hybridoma supernatant. Then, they were stained with an anti-mouse Ab conjugated to Cy5 and analyzed by flow cytometry. Four independent lines of wild-type (■) MEFs and four BH<sup>-/-</sup> lines were analyzed. Error bars represent the SD between independent lines. Results are representative of at least three independent experiments.

(or donkey anti-rat) F(ab')<sub>2</sub> conjugated to Cy5 (Jackson ImmunoResearch Laboratories). For staining cells isolated from spleens, AF6-88.5 (anti-H-2K<sup>b</sup>) and KH95 (anti-H-2D<sup>b</sup>) Abs conjugated to a fluorophore were used according to the manufacturer's directions (BD Biosciences). The cells were then analyzed by flow cytometry on a FACSCalibur apparatus (BD Biosciences) with FlowJo software (Tree Star).

### Tissue culture and transfections

MEFs were cultured at 37°C and 10% CO<sub>2</sub> in DMEM plus 20% FCS. Transfections were performed with various constructs using Fugene 6 (Roche Molecular Biochemicals) according to the manufacturer's instructions.

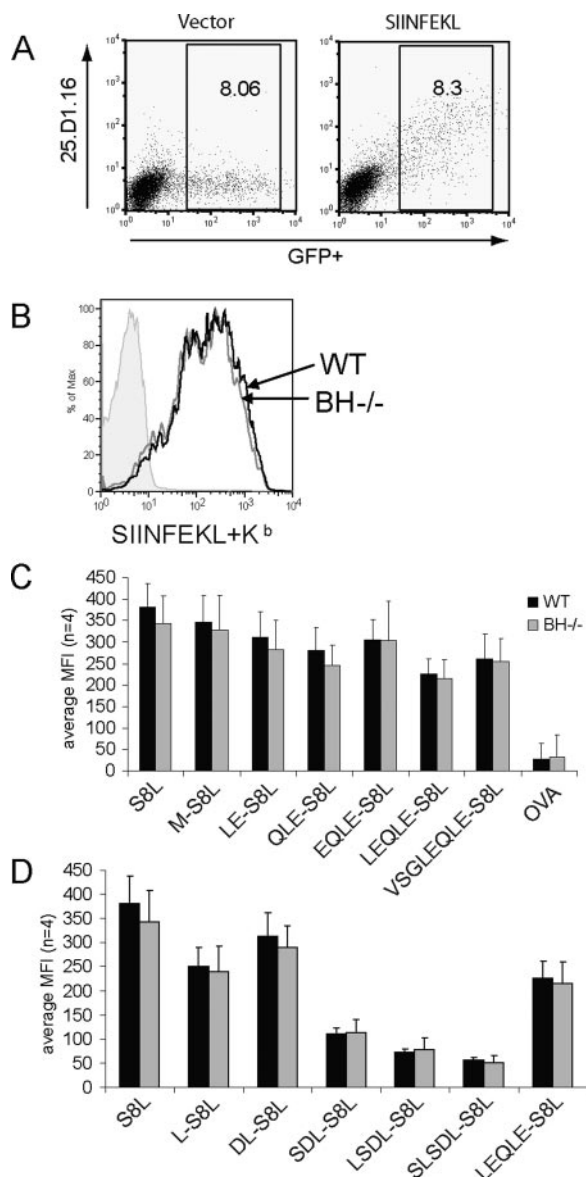
## Results

### BH<sup>-/-</sup> MEFs present peptide as efficiently as wild-type MEFs

To examine the ability of cells from BH-deficient mice to present peptides, we first generated and analyzed MEFs. Multiple-independent MEF lines were established from BH-deficient and control mice. The mutant and wild-type lines were indistinguishable in terms of their morphology and growth characteristics (data not shown).

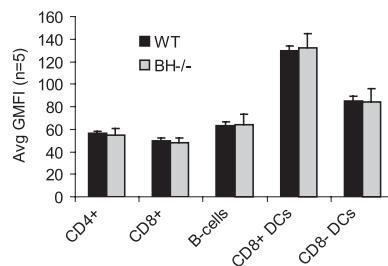
For MHC class I molecules to be transported to the cell surface, they must first bind a peptide in the ER. Surface expression of MHC class I molecules is therefore an indirect measure of peptide supply. Expression of H-2K<sup>b</sup> (stained with Y3) and of all MHC class I alleles (stained with M1/42) were similar among four independently derived BH-deficient and wild-type MEFs (Fig. 1), indicating that BH is neither essential for the generation of peptides nor rate limiting for destroying epitopes that can be presented by MHC class I molecules in these cells.

We next examined the ability of MEFs to generate specific MHC class I-presented peptides. Because BH is a cytosolic peptidase, we examined the ability of the mutant MEFs to generate presented peptides from precursors expressed in the cytosol. For these experiments, MEFs were transfected with a series of constructs encoding GFP and ubiquitin-fusion minigenes. In these plasmids, the C terminus of ubiquitin is fused to an N-extended form of the SIINFEKL peptide. Cleavage by ubiquitin C-terminal hydrolases (41) releases the peptide of interest with no initiating methionine, thus generating peptides similar to those generated by the proteasome. Subsequent trimming of the N-terminal residues releases SIINFEKL, which if presented, can be detected bound to surface H-2K<sup>b</sup> molecules by 25.D1.16 staining. The natural upstream sequence of SIINFEKL (LEQLE-SIINFEKL) was tested because it has been used in previous experiments and is known to



**FIGURE 2.** SIINFEKL presentation on MEFs from peptide precursors. *A*, MEFs were transfected with a construct expressing GFP alone (Vector), or GFP and SIINFEKL peptide conjugated to the C terminus of ubiquitin (SIINFEKL). Twenty-four hours after transfection, cells were stained with 25.D1.16 and analyzed by flow cytometry by gating on cells with comparable amounts of GFP. The percentage of transfected cells was comparable between constructs. *B*, MEFs from wild-type (black dashed) and BH-deficient (gray) embryos were transfected with plasmids containing GFP and SIINFEKL and analyzed as in *A*. The cells were then compared for SIINFEKL presentation. *C*, Wild-type (■) and BH-deficient (□) MEFs were transfected as in *A* with a variety of peptide precursors varying in length, containing the natural upstream sequence of SIINFEKL peptide. After 24 h, cells were analyzed by flow cytometry and the mean fluorescence intensity (MFI) of 25.D1.16 staining from four independent MEF lines of each genotype was averaged. Error bars represent the SD between the MFI of the four MEF lines. *D*, Wild-type (■) and BH<sup>-/-</sup> MEFs (□) were transfected and analyzed as in *C* with SIINFEKL precursors containing the N-terminal sequence of the VSV-NP peptide.

be trimmed in live cells (5). MEFs were transfected with various constructs and analyzed by flow cytometry after 24 h by gating on cell populations expressing comparable amounts of GFP (Fig. 2*A*). Three independent MEF mutant and wild-type cell lines were used in all experiments to evaluate the variability between different lines that was unrelated to BH deficiency.



**FIGURE 3.** MHC class I presentation on BH-deficient splenocytes is as efficient as wild type. Splens from sex-matched mice were collected and splenocytes were then stained with Abs for CD4, CD8, B220, and H2-K<sup>b</sup>. Separate staining for dendritic cell populations included staining for CD8, CD11c, CD86, and H2-K<sup>b</sup>. After staining, cells were collected by flow cytometry, enumerated, and analyzed for MHC class I expression of various cell populations. H2-K<sup>b</sup> expression on CD4<sup>+</sup>, B220<sup>-</sup>, CD8<sup>-</sup> cells was the same between wild-type (■) and BH<sup>-/-</sup> (□) mice. The same was true for CD8<sup>+</sup>, CD4<sup>-</sup>, B220<sup>-</sup> cells, as well as CD8<sup>+</sup>, CD4<sup>-</sup>, and B220<sup>-</sup> cells. Dendritic cells were gated based on CD86<sup>high</sup>, CD11c<sup>high</sup> expression. H2-K<sup>b</sup> presentation on the CD8<sup>+</sup> and CD8<sup>-</sup> subsets of this cell population was comparable between genotypes. Error bars represent the variation in the geometric MFI of five mice per genotype. Data are representative of at least three independent experiments.

The level of SIINFEKL presentation from the minimal epitope (i.e., with no extra flanking residues) was similar between BH-deficient, and wild-type MEFs (Fig. 2, *B* and *C*). This indicated that BH was not destroying this epitope by trimming it to a size that was too short to stably bind H-2K<sup>b</sup>. It also indicated that BH deficiency does not positively or negatively impact steps after peptide generation in the MHC class I pathway (e.g., peptide transport, etc.).

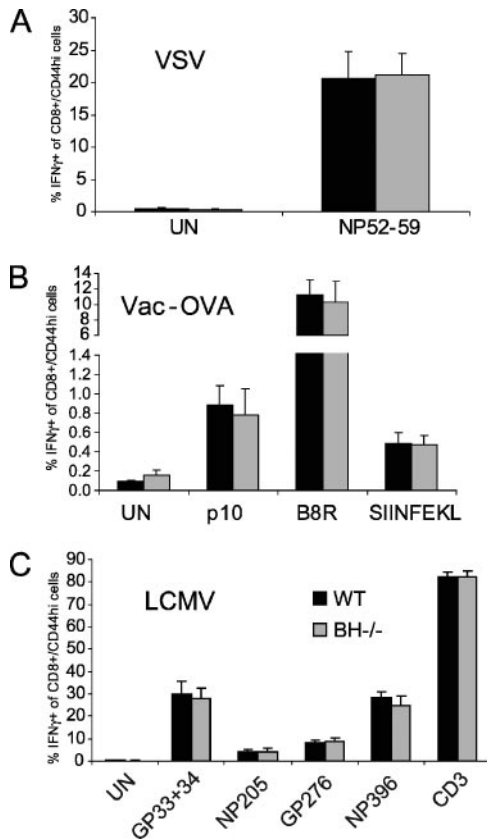
Levels of SIINFEKL derived from N-extended precursors or full-length OVA were presented similarly in BH<sup>-/-</sup> and wild-type MEFs, regardless of precursor length (Fig. 2*C*). These data suggest that BH is not essential for trimming the elongated SIINFEKL precursors and that BH is not required for trimming the residues M, L, E, or Q.

Because BH was initially identified as an enzyme that trimmed the upstream sequence of the VSV-NP peptide (7), a second series of SIINFEKL precursors were made as ubiquitin fusion proteins, in which the natural upstream sequence of SIINFEKL was replaced with the upstream sequence of SLSDL that flanks the VSV epitope RGYVYQGL. These new constructs were then used in the same assay to determine whether BH was important for trimming this peptide sequence.

Wild-type and BH-deficient MEFs presented the same amount of SIINFEKL from the VSV precursors (Fig. 2*D*), indicating that BH is not required for trimming the SLSDL sequence which precedes the VSV NP52–59 epitope. The reduction in presentation of SIINFEKL from the SLSDLSIINFEKL construct as compared with the normal LEQLESIIINFEKL sequence (Fig. 2) is presumably due to the content or sequence of the flanking N-terminal amino acids rather than the length of the N-terminal extension (SIINFEKL was underlined in the previous sentence to emphasize the commonality between peptides.) In any case, this difference in presentation is unrelated to the presence or absence of BH.

#### *Lymphocyte ratios and MHC class I levels in BH<sup>-/-</sup> mice are normal*

We examined the number and ratios of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the spleen and lymph nodes of BH<sup>-/-</sup> mice, because changes in thymic Ag presentation could influence the composition of the peripheral T cell pool. In addition, we examined the levels of MHC

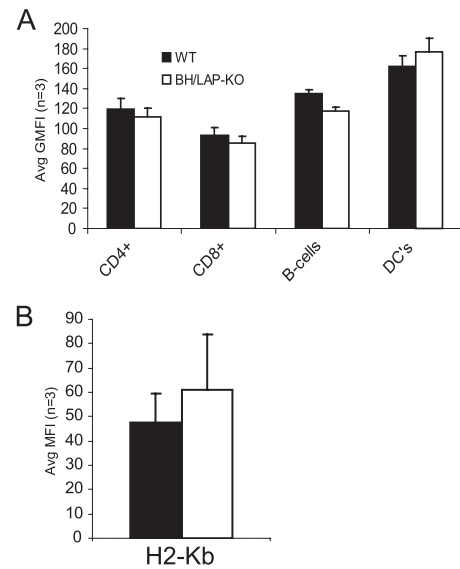


**FIGURE 4.** Intracellular IFN- $\gamma$  staining of peptide-specific T cells. Spleen cells from wild-type (■) or BH<sup>-/-</sup> (□) mice were stimulated on: *A*, day 7 for VSV; *B*, day 7 for recombinant vaccinia; or *C*, day 9 for LCMV. After isolation, splenocytes were stimulated for 5 h with: *A*, VSV peptide NP52–59; *B*, vaccinia peptide p10 or B8R, or with SIINFEKL peptide; or *C*, LCMV peptides gp33, np205, gp276, and np396. They were then surface stained with anti-CD8 and anti-CD44, and intracellularly stained with anti-IFN- $\gamma$ . Numbers indicate the average percentage of CD8/CD44<sup>high</sup> T cells that were IFN- $\gamma$ -positive from five mice. Error bars represent the SD within each group. Data represent results from at least two experiments each. There was no significant difference between BH<sup>-/-</sup> and wild-type mice in their response to any of the epitopes tested.

class I on the surface of major lymphocyte populations in the spleen to determine whether BH is required for normal levels of peptide presentation. The number and frequency of CD8<sup>+</sup> T cells in lymph nodes and spleen were equal to those in wild-type mice (data not shown), suggesting that BH is not required for thymic selection. MHC class I expression on CTL (CD8<sup>+</sup>, CD4<sup>-</sup>, B220<sup>-</sup>), Th cells (CD4<sup>+</sup>, CD8<sup>-</sup>, B220<sup>-</sup>), B cells (B220<sup>+</sup>, CD4<sup>-</sup>, and CD8<sup>-</sup>), and dendritic cells (CD11c<sup>+</sup>, CD86<sup>+</sup>, CD8<sup>+</sup> or CD8<sup>-</sup>) from BH<sup>-/-</sup> mice presented MHC class I as efficiently as wild-type mice (Fig. 3), indicating that BH is not required for MHC class I presentation in these cell populations.

#### Presentation of viral epitopes is not altered in BH<sup>-/-</sup> mice

Because BH has previously been shown to act on precursors of viral epitopes to generate the correct peptide *in vitro* (7), it seemed possible that BH may have a critical role in generating MHC class I-presented peptides from viral proteins during an infection *in vivo*. If so, then BH<sup>-/-</sup> mice would mount a reduced CTL response to these epitopes, as has been observed, e.g., in ERAP1-deficient mice (15). Therefore, BH mice were infected with VSV, LCMV, or Vac-OVA, and the CTL response to viral epitopes was



**FIGURE 5.** MHC class I presentation on BH/LAP-deficient cells. *A*, Spleens were collected from sex-matched mice and wild-type (■) and BH/LAP-knockout (KO) (□) splenocytes were then stained and analyzed as in Fig. 4. *B*, Wild-type (■) and BH/LAP-KO (□) MEFs were stained for H2-K<sup>b</sup>. On all cell populations, MHC class I presentation was comparable between genotypes. Error bars represent the variation in the GMFI of five mice per genotype (*A*), or in the MFI of three independent MEF lines (*B*). Data are representative of at least three independent experiments.

analyzed at the peak of the immune response to each infection by intracellular cytokine staining for IFN- $\gamma$ .

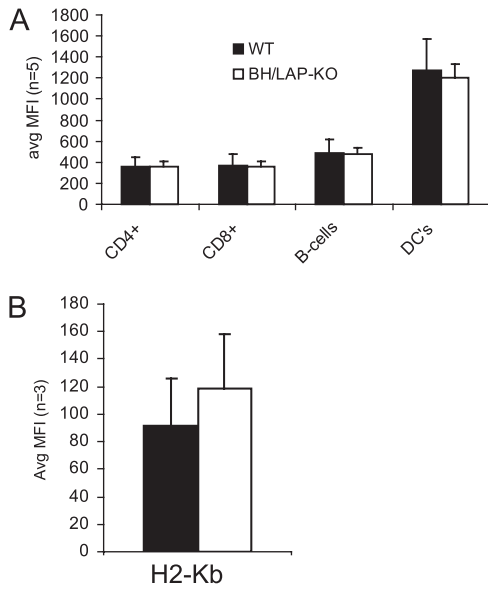
The VSV-NP epitope RGYVYQGL, which binds H-2K<sup>b</sup> (47), elicited similar responses in the BH<sup>-/-</sup> and wild-type control mice (Fig. 4A), despite the observation that BH is important for the trimming of this epitope *in vitro* (7). The CTL responses to SIINFEKL, P10, and B8R epitopes from Vac-OVA were equivalent in BH<sup>-/-</sup> and wild-type mice (Fig. 4B), and in LCMV-infected mice, responses to gp33 plus 34 (which contains both H-2K<sup>b</sup> and H-2D<sup>b</sup>-binding peptides), gp276 which binds H-2D<sup>b</sup>, NP205 which binds H-2K<sup>b</sup>, and the H-2D<sup>b</sup>-binding NP396 were comparable in BH<sup>-/-</sup> and wild-type mice (Fig. 4C). Therefore, BH does not play an essential role in generating any of these eight peptides or conversely in limiting their presentation.

#### Generation of mice and cells lacking BH and LAP

The finding that BH<sup>-/-</sup> mice and LAP<sup>-/-</sup> mice (21) individually showed no detectable change in Ag presentation raised the possibility that the functions of these aminopeptidases were largely redundant. To investigate this possibility, we crossed BH-deficient and LAP-deficient mice to generate animals deficient in both peptidases. The double-deficient mice were viable and had no obvious phenotypic change, aside from a slight dwarfism previously reported for the BH-deficient mice (39). MEFs derived from the double-deficient mice had the same morphology and growth rate as wild-type MEFs.

#### BH and LAP together are not essential for normal levels of MHC class I peptide generation in lymphocytes

We analyzed the cellular composition in the spleen and thymus of BH/LAP-deficient mice. There were similar numbers of splenocytes in the BH/LAP-deficient and wild-type mice, and the percentage of CD8<sup>+</sup> T cells, CD4<sup>+</sup> T cells, B cells, and dendritic cells was unchanged (data not shown).



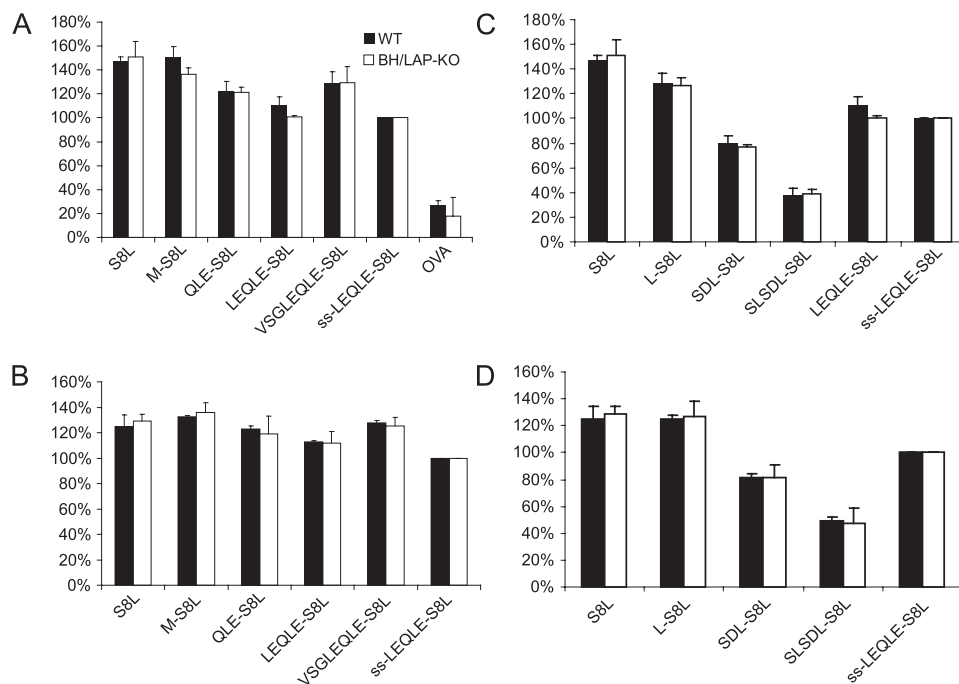
**FIGURE 6.** MHC class I presentation on stimulated BH/LAP-deficient cells. *A*, Sex-matched mice were injected i.p. with 200  $\mu$ g of poly I:C. After 24 h, the spleens were collected and splenocytes were then stained and analyzed as in Fig. 4. *B*, Wild-type (■) and BH/LAP-KO (□) MEFs were stained for H2-K<sup>b</sup> levels 24 h after treatment with murine IFN- $\gamma$ . On all cell populations, MHC class I presentation was comparable between genotypes. Error bars represent the variation in the GMFI of five mice per genotype (*A*), or in the MFI of three independent MEF lines (*B*). Data are representative of at least three independent experiments.

We stained the various splenocytes with Abs against MHC class I molecules. There was no overall reduction or increase in cell surface H-2K<sup>b</sup> or H-2D<sup>b</sup> molecules compared with wild-type mice. There were also no changes in the surface expression of these molecules on different cell subpopulations in the spleen, including T, B, and dendritic cells (Fig. 5*A*). Similarly, BH/LAP-deficient and wild-type MEFs expressed similar levels of MHC class I molecules (Fig. 5*B*). Therefore, eliminating these two major cytosolic peptidases does not detectably reduce or increase the overall supply of peptides to MHC class I molecules.

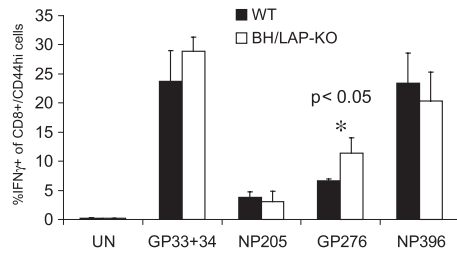
LAP has been shown to be inducible by IFN (18, 21). Although the absence of LAP alone does not affect the levels of MHC class I presentation on living cells in the presence of IFN- $\gamma$  (21), it was possible that the absence of two aminopeptidases under these conditions might result in a change in the MHC class I peptide pool. Therefore, we examined the levels of MHC class I on splenocyte populations after injection with poly I:C, which stimulates the production of type I IFNs. Poly I:C treatment increased MHC class I levels to similar levels on splenocytes from BH/LAP-deficient mice and wild-type mice (Fig. 6*A*). Similarly, we treated MEFs with IFN- $\gamma$  and examined MHC class I levels after 24 h. As with the splenocytes, the increase in MHC class I levels was similar on BH/LAP-deficient and wild-type MEFs (Fig. 6*B*).

*BH/LAP-deficient MEFs can generate SIINF EK L from peptide precursors*

We next investigated whether the loss of both BH and LAP affected the ability of MEFs to present specific peptides. BH/LAP-deficient and wild-type MEFs generated the same amount of SIINFKEL-H-2K<sup>b</sup> complexes when transfected with the ubiquitin-SIINF EK L mini construct (Fig. 7*A*), which produces the mature SIINF EK L epitope in



**FIGURE 7.** SIINF EK L presentation on MEFs from peptide precursors. MEFs from wild-type (■) and BH/LAP-deficient (□) embryos were transfected in each experiment with constructs expressing GFP and a SIINF EK L peptide conjugated to the C terminus of ubiquitin. Twenty-four hours after each transfection, cells were stained with 25.D1.16 and analyzed by flow cytometry. Cells were gated based on comparable amounts of GFP expression and SIINF EK L+K<sup>b</sup> presentation was compared. In each case, MFI values were normalized based on the presentation of SIINF EK L from an ER-targeted SIINF EK L precursor control by each MEF line. *A*, MEFs were transfected with full-length OVA or SIINF EK L precursors with the natural N-terminal OVA sequence. *B*, IFN- $\gamma$  was added to the medium 8 h after transfection with the same SIINF EK L constructs as in *A*. *C*, MEFs were transfected with constructs expressing SIINF EK L preceded by the N-terminal VSV sequence. *D*, Eight hours after transfection with the constructs used in *C*, IFN- $\gamma$  was added to the culture medium.



**FIGURE 8.** Intracellular IFN- $\gamma$  staining of peptide-specific T cells. Spleen cells from wild-type (■) and BH/LAP-KO (□) mice were stimulated on day 9 after LCMV infection. After isolation, splenocytes were stimulated for 5 h with gp33, np205, gp276, or np396 peptide. They were then surface stained with anti-CD8 and anti-CD44 and intracellularly stained with anti-IFN- $\gamma$ . Numbers indicate the average percentage of CD8 $^+$ /CD44 $^{\text{high}}$  cells that were IFN- $\gamma$  positive from three mice. Error bars represent the SD within each group. Results represent data from at least two experiments. BH/LAP-KO mice had significantly higher T cell responses ( $p < 0.05$ ) to GP276 peptide, but not to other peptides.

the cytosol after ubiquitin hydrolysis. Therefore, the loss of both peptidases did not increase presentation through reduced destruction of SIINFEKL.

These cells were also transfected with N-extended precursors of SIINFEKL. As a control, the cells were also transfected with an ER-targeted SIINFEKL precursor that was used as a benchmark to normalize the expression of SIINFEKL from cytosolic precursors in the different MEF lines (ss-LEQLE-S8L); this facilitated comparisons by factoring out any cell-to-cell variability in the maximum level of Ag presentation. There was no reduction or increase in the generation of SIINFEKL plus H-2K $^b$  complexes from precursor containing the natural upstream SIINFEKL sequence LEQLE under normal conditions (Fig. 7A), or after treatment with IFN- $\gamma$  (Fig. 7B) even though overall levels of SIINFEKL presentation were increased after IFN treatment.

Adding the VSV-NP N-terminal sequence to the SIINFEKL peptide resulted in a change in SIINFEKL presentation compared with the natural sequence, but SIINFEKL presentation was still comparable between BH/LAP-deficient MEFs and wild-type MEFs (Fig. 7C). After treating the MEFs with IFN for 24 h, SIINFEKL presentation from all the VSV precursors increased on BH/LAP-deficient and wild-type MEFs, but there was no significant difference in SIINFEKL presentation between wild-type and BH/LAP knockout cells (Fig. 7D). Thus, the double-deficient MEFs are as capable as wild-type or single-deficient MEFs in their ability to trim and present these N-extended precursors.

#### *CD8 T cell responses to one viral epitope are selectively increased in BH and LAP-deficient mice*

BH/LAP-deficient mice were also examined for their ability to respond to various viral Ags during an infection. Although CD8 $^+$  T cells in wild-type and double knockout mice responded equivalently to the VSV, Vac-OVA, and many LCMV epitopes (Fig. 8 and data not shown), the CTL response to the LCMV gp276 epitope was significantly higher in double knockout mice (Fig. 8). This suggests that BH and LAP normally reduce presentation of this particular peptide to CTL. Because this effect is not seen in mice lacking only BH or LAP, the ability of these two peptides to reduce the presentation of this epitope is presumably functionally redundant. In contrast, responses to three other LCMV epitopes (gp33 plus 34, np207, and np396), and four epitopes from other viruses (P10, B8R, SIINFEKL, and NP52–59), in the double-de-

ficient mice were the same as those of single-deficient and wild-type mice.

## Discussion

Cytosolic aminopeptidases are thought to shape the repertoire of MHC class I-presented peptides by removing extra N-terminal flanking residues from long antigenic precursors generated by proteasomes (1, 19, 40). They also may destroy peptides by trimming them to a size below the minimum necessary to be transported by TAP and bind to MHC class I molecules. However, the contribution of individual cytosolic aminopeptidases to these processes is still unclear.

BH is one of the cytosolic aminopeptidases that has been proposed to contribute to MHC class I peptide presentation because it was identified as a major trimming activity of the VSV-NP epitope in cell lysates treated with proteasome inhibitors. As well, treatment of cells with inhibitors of BH such as AAF-CMK (7) and E-64 (32) reduce presentation of some MHC class I epitopes. However, because AAF-CMK and E-64 block the activity of peptidases other than BH, these studies do not unequivocally demonstrate that BH is involved in Ag presentation in living cells.

To more precisely define the contribution of BH to Ag presentation, we analyzed cells and mice lacking BH. BH-deficient MEFs showed no defect or enhancement in presenting SIINFEKL from peptide precursors containing the natural upstream sequence (LEQLE) of SIINFEKL or the SLSDDL flanking sequence from the VSV-NP epitope. Thus, although BH was previously implicated in the trimming of the N-terminal sequence SLSDDL of the VSV-NP epitope (7), it is not required *in vivo*. It is possible that BH is required for trimming other sequences. However, BH-deficient MEFs had normal surface expression of MHC class I molecules, indicating that BH does not play an essential role in generating or destroying the majority of peptides that bind to MHC class I in these cells.

The presentation of SIINFEKL was negatively affected when it was preceded by the SLSDDL sequence irrespective of whether the MEFs were wild type or mutant. This reduction in presentation was detectable with the first added residue (L) and decreased with every additional amino acid that was added to the SIINFEKL sequence (Fig. 2), with the difference between SLSDDL and LEQLE being the most pronounced. This is yet another example of the influences that flanking sequences can impose upon the MHC class I presentation of epitopes (52–56). Presumably, the enzymes responsible for removing the N-terminal sequences are more active against LEQLE than the SLSDDL sequence. In any case, we can conclude that BH is not essential for this trimming process.

Because the function of ERAAP (ERAAP) can influence lymphocyte development (16), we examined T cell, B cell, and other leukocyte populations in the periphery and thymus of BH-deficient mice. The mutant animals had normal total numbers and percentages of B cells, dendritic cells, and CD8 and CD4 T cells in their spleens and lymph nodes, showing that BH is not required for the development or maintenance of these leukocytes.

BH-mutant mice mounted normal CD8 T cell responses *in vivo* to all eight viral epitopes examined, from three different viruses. Based on these findings, we conclude that BH does not play a major role either in generating or destroying the MHC class I-presented epitopes we examined. Again, it remains possible that BH is responsible for generating or destroying a minor population of peptides.

Given our results, it seems likely that multiple intracellular aminopeptidases have redundant functions, both in their main cellular function of degrading proteasomal products into amino acids,

and in the generation of MHC class I peptides from antigenic precursors. Consistent with this notion, purified puromycin-sensitive aminopeptidase as well as BH has been shown to trim N-extended VSV-NP precursors in vitro (7). Functional redundancy may also explain the lack of an effect on MHC class I peptide presentation observed in cells and mice deficient in IFN-inducible LAP (21).

Functional redundancy among aminopeptidases may explain why BH/LAP-deficient mice showed a significantly higher CTL response to a viral epitope (gp276, presented by H-2D<sup>b</sup>). Because the responses were higher in the absence of the aminopeptidases, the implication is that they normally destroy this epitope, presumably by trimming it below the length needed to bind to the MHC class I molecule. BH and LAP can both trim peptides to sizes below 7 residues (7, 18). In any case, because this change in the immune response was not seen in mice deficient for either BH or LAP alone, the clear implication is that BH and LAP overlap in function.

Outside of the change in presentation of gp276, the double knockout mice showed no additional abnormalities in Ag presentation that we could detect. Surface expression of MHC class I on MEFs, presentation of defined epitopes by MEFs, and absolute cell numbers and cell ratios in vivo were all similar to wild-type cells and mice. In addition, the CTL response to seven other epitopes was identical in the wild-type and mutant mice. Because a portion of the trimming of antigenic precursors occurs in the cytosol (13), these findings indicate that there is at least one other dominant and/or redundant cytosolic aminopeptidase involved in Ag presentation. It is possible that loss of BH and/or LAP results in compensatory changes in the expression of other redundant peptidases. However, we have not detected such changes, at least by examining a number of other well-known cytosolic enzymes. In any case, even if such effects were to occur, they would still point to redundant functions between peptidases.

It is not clear why BH and LAP deficiency affects the presentation of gp276, but not the other peptides we examined. The gp276 epitope is an exceptionally long one, 11 aa compared with the 9 or 10 residues that are more common among MHC class I epitopes. Because most peptidases preferentially trim relatively short peptides (14), perhaps relatively few aminopeptidases are able to trim gp276; if so, it is possible that eliminating just two aminopeptidases leads to a detectable effect on Ag presentation of this epitope but not others. Alternatively, BH and LAP may be more active against the gp276 sequence than other aminopeptidases. Interestingly, the same gp276 epitope is also presented at higher levels in ERAP1<sup>-/-</sup> mice (15), suggesting that multiple enzymatic activities in different cellular compartments shape the MHC class I peptide repertoire. It is also formally possible that BH and LAP double deficiency affect the T cell repertoire in ways that selectively reduces the response to the gp276 epitope.

Together, the data suggest that BH, LAP, and ERAP1 (and potentially other) enzymes contribute to shaping the MHC class I peptide repertoire in many common and potentially some unique ways. Any unique effects presumably reflect differences in the specificity of the enzymes for N-terminal sequence, or perhaps for the internal amino acid sequence of the peptide precursor. The identity of the peptidases involved in this process and their specificity will be important to define in future studies.

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## Disclosures

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