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Processing of Recombinant *Listeria monocytogenes* Proteins for MHC Class I Presentation Follows a Dedicated, High-Efficiency Pathway

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CD8+ T lymphocytes recognize short peptides of \(~8–10\) aa bound to MHC class I molecules (pMHC) on the surface of APCs. These peptides can be generated from either endogenous proteins synthesized by the biosynthetic machinery of the presenting cell or from exogenously sourced proteins. Because much of the research characterizing the MHC class I processing pathway has focused on endogenously synthesized proteins, it is not known whether differences exist in the processing pathway followed by endogenously synthesized versus exogenously sourced proteins. To highlight potential differences in the processing of endogenous versus exogenous proteins, we developed a model system to measure the efficiency of pMHC generation from nearly identical recombinant proteins expressed from vaccinia virus and *Listeria monocytogenes*. In these experiments, we uncovered a striking difference in the way recombinant *Listeria* Ags are processed and presented when compared with endogenously synthesized viral proteins. Specifically, we find that pMHC production from secreted *Listeria* proteins occurs at the same rate, independent of the cellular half-life of the protein from which it is derived, whereas the rate of pMHC production from endogenously synthesized viral proteins is absolutely dependent on its protein half-life. Accordingly, our data demonstrate the existence of a distinct and highly efficient MHC class I presentation pathway used for the processing of at least some exogenously synthesized proteins. *The Journal of Immunology*, 2013, 190:000–000.

Most, although not all, peptides presented by MHC class I molecules (pMHC) are generated by the degradation of proteins via the ubiquitin (Ub) proteasome system. A general feature of this pathway is that, for a given protein, increasing the number of molecules degraded will result in a proportionally greater supply of peptides available for presentation on MHC class I molecules. Consequently, the supply of peptides available for presentation by MHC class I molecules can be increased by radically shortening the cellular half-life of a protein through the introduction of amino acid sequences that target the protein for degradation (1–3). This approach has been used in several studies and, in virtually every system tested, increasing degradation of a given protein has resulted in a concomitant increase in surface expression of pMHC class I molecules containing peptides derived from that protein (3, 4).

We have previously shown that there is an inverse correlation between protein half-life and pMHC surface levels using a recombinant vaccinia virus (rVV) expression system (4). By measuring the kinetics of surface pMHC expression, we showed that decreasing the cellular half life of a substrate protein results in a concomitant increase in pMHC generation. Additionally, we determined that the average efficiency of surface pMHC expression for a given peptide generated from a recombinant protein is \(~1\) pMHC per 3000 protein molecules degraded. An important observation made during the course of these studies was that highly similar proteins can be processed with different efficiencies. Specifically, pMHC complexes are generated more efficiently from a cellular pool of recombinant influenza nucleoprotein (NP) with a \(t_{1/2}\) of \(~70\) min than from a nearly identical protein designed to be degraded in a near cotranslational manner (\(t_{1/2}\) of \(~10\) min).

The only other study published to date examining the efficiency of pMHC generation is that of Pamer and colleagues (5), which measured pMHC generation from proteins secreted by *Listeria monocytogenes*. *Listeria* is an intracellular Gram-positive bacterium that infects primarily professional phagocytic cells such as macrophages and dendritic cells. Upon uptake, *Listeria* initially resides within the host cell phagosome. Within minutes of formation of the phagosome, the *Listeria* begin to secrete virulence factors such as listeriolysin O and phospholipase C that results in lysis of the phagosomal membrane and bacterial escape into the host cell cytosol. As a result, any proteins secreted by the bacterium have direct access to the MHC class I Ag processing pathway of the host cell (6). This ability of *Listeria* to secrete proteins directly into the cytosol of infected cells has made it a unique tool for studying class I processing of secreted bacterial proteins.

Determining processing efficiency is technologically challenging. Any values determined experimentally should be considered, at best, approximations of absolute values that might be associated with a particular model system. With that said, the studies by Pamer and colleagues (5, 7) showed that one pMHC is generated for every \(\sim 4–35\) protein molecules degraded, an efficiency 2–3 orders of magnitude greater than that measured for recombinant proteins expressed from vaccinia virus. It is unlikely that such
large differences in processing efficiency are the result of experimental variability. What could account for the difference in processing efficiency between Listeria and vaccinia virus? One possibility is that, whereas the Listeria study examined presentation of peptides derived from native bacterial proteins, the vaccinia study measured presentation of a model peptide determinant generated from a recombinant protein. This would suggest that the differences in efficiency observed between the Listeria and vaccinia studies reflect differences inherent to the specific proteins and/or peptides used for these studies. To test this, we designed a novel system for expressing and secreting recombinant proteins from Listeria, which would allow us to determine the efficiency of pMHC production from a stable and rapidly degraded fusion protein in a manner analogous to our previous study using rVV.

Remarkably, we report in this study that the rate of pMHC generation for recombinant protein expressed by Listeria did not change, regardless of the cellular half-life of the protein. Furthermore, the efficiency of pMHC generation from recombinant Listeria proteins was at least 19-fold more efficient than that for the same recombinant proteins expressed by vaccinia. Taken together, our results suggest that proteins secreted from Listeria that are processed for MHC class I presentation follow, almost exclusively, a high efficiency processing pathway that is only accessed by a subset of endogenously synthesized proteins.

Materials and Methods

Cell line cultures

L929 cells, X63-Ag GM-CSF–expressing cells, and the macrophage-like cell line BMA 3.1A7 (BMA3; provided by Ken Rock, University of Massachusetts Medical School, Worcester, MA) were grown in IMDM (HyClone) supplemented with 10% heat-inactivated FBS (Cellgro) and 1× Glutamax (Invitrogen). All cells were maintained at 37°C in 7% CO₂ atmosphere.

Bone-marrow macrophage and dendritic cell generation

Bone marrow cells were isolated from the femurs and tibiae of C57BL/6J mice (The Jackson Laboratory). Bone marrow–derived macrophages (BMMds) and dendritic cells (BMDCs) were generated by incubating bone marrow cells in growth medium consisting of IMDM with 10% heat-inactivated FBS, 1× Glutamax, 1× penicillin and streptomycin (Cellgro), 25 mM HEPES (Life Technologies/Invitrogen), and supplemented with 14% L929 cell–conditioned culture medium for BMMds or 0.72 mM 2-ME (Sigma-Aldrich) and 2% X63-Ag GM-CSF–conditioned culture medium for BMDCs. BMMds were used after 6–8 d in culture, whereas BMDCs were used after 8–9 d in culture. Mice were used in accordance with Institutional Animal Care and Use Committee guidelines. The State University of New York Upstate Committee on the Humane Use of Animals approved all protocols.

Recombinant Listeria strains

Generation of ActA-deficient Listeria expressing the stable recombinant protein Ub-R-NP-S-FLAG/NF-L1177 (AU-NP-S-FLAG) AU-NP-S-FLAG (invitrogen) from ActA-deficient Listeria has been previously described (8). Rapidly degraded AU-arginine (R)-NP-S-FLAG was generated as follows. DNA encoding Ub-R-NP-S-ARDG from rVV was amplified from rVV expressing Ub-R-NP-S-ARDG (Invitrogen) and supplemented with 1% L929 cell–conditioned culture medium for BMMds or 0.72 mM 2-ME (Sigma-Aldrich) and 2% X63-Ag GM-CSF–conditioned culture medium for BMDCs. BMMds were used after 6–8 d in culture, whereas BMDCs were used after 8–9 d in culture. Mice were used in accordance with Institutional Animal Care and Use Committee guidelines. The State University of New York Upstate Committee on the Humane Use of Animals approved all protocols.

PCR sequences were extended at the 3’ end to add nucleotides encoding SINFEKL-ARDG. Both PCR products were digested with AflIII and XmaI, ligated into pP2L, and conjugated into Listeria as described previously (8). All Listeria strains used in this study express a nonsecreted form of eGFP or mCherry encoded by the pNF8 plasmid (provided by Bruce Applegate, Purdue University, West Lafayette, IN) (8, 9).

Listeria and rVV infections

Listeria infection of BMA3 cells was as previously described (8). Briefly, BMA3 cells were infected with log-phase Listeria at a multiplicity of infection (MOI) of 20 for 1 h. Cells were washed, resuspended in IMDM containing 5 µg/ml gentamicin (Cellgro) to kill any remaining extracellular bacteria (4), and incubated at 37°C for the duration of the experiment. This protocol was used for Listeria infection of BMMds and BMDCs except that cells were lifted using 2 mM EDTA (Life Technologies/Invitrogen) in PBS, and BMMds were infected at an MOI of 10.

Infection of BMA3 cells with rVV was as previously described (8). Briefly, BMA3 cells were resuspended in HBSS (Cellgro) containing 0.1% BSA (Fisher Scientific) and rVV at an MOI of 10 for 15 min with constant agitation, then resuspended in IMDM and incubated at 37°C for the duration of the experiment.

Extracellular (IMDM) secretion rates

To determine secretion rates of recombinant proteins, 1 × 10⁶ CFU log-phase Listeria were washed in ice-cold IMDM, resuspended in 5 ml IMDM at 37°C, and grown on an orbital shaker at 37°C at 225 rpm for 2 h. Aliquots were taken at 0.5, 1, and 2 h postinoculation and Listeria were removed by centrifugation. Culture supernatants were stored at –80°C for use in Western blot analysis.

Intracellular secretion rate

Adherent BMA3 cells were infected with trypsin (Cellgro), resuspended in IMDM, and treated with 5 µM epoxomicin (Enzo Life Sciences) or DMSO vehicle (American Type Culture Collection) for 30 min at 37°C. BMA3 cells were then washed in IMDM to remove excess drug and infected with Listeria. Three hours postinfection, aliquots were lysed at 4 × 10⁶ cell equivalents/ml using RIPA buffer (150 mM NaCl [Fisher Scientific], 50 mM Tris [pH 8.0; Invitrogen], 1 mM EDTA [Invitrogen], 0.1% SDS [Life Technologies/Invitrogen], 0.5% deoxycholate [Sigma-Aldrich], 1% Igepal CO-630 [Sigma-Aldrich]). Lysates were cleared of insoluble material by centrifugation and stored at –80°C for use in Western blot analysis.

Western blot analysis

The following Abs were used for western blot analysis: M2 anti-FLAG (Sigma-Aldrich), anti-β-actin clone AC-15 (Sigma-Aldrich), culture supernatant from the anti-influenza NP hybridoma H19-S24-C16 (provided by Nancy Freitag, University of Illinois at Chicago College of Medicine, Chicago IL), which has constitutive expression of pRFA-regulated genes, such as influenza NP, and AU-NP-S-FLAG (10). Aliquots of GI455S (AU-NP-S-FLAG expressed from NF-L1177) for use as a protein standard were prepared from supernatants of overnight cultures and stored at –80°C. Absolute concentrations of GI455S were determined by comparing with a known NP standard prepared from purified influenza virus A/Puerto Rico/8/34 (Charles River).

Fluorescence

BMA3 cells and BMDCs were incubated in 2.4G2 hybridoma supernatant to block CD16/CD32 FCy receptors (7) and stained with Alexa Fluor 647 (Invitrogen)–conjugated 25-D1.16 mAb (8). BMDCs were additionally stained with PE–anti-CD11c (BioLegend) and gated on CD11b–CD11c– cells for all analyses. BMMds were prepared similarly to BMA3 cells with the exception that 20% mouse serum (Sigma-Aldrich) was added during the blocking step. Fluorescence
was determined using an LSR II flow cytometer (BD Biosciences). Mean fluorescent intensity was correlated to the number of surface K\textsuperscript{b}-SIINFEKL molecules using Dako FluoroSpheres as described previously (8). Data were analyzed using FlowJo software (Tree Star).

Results

N-end rule degrons secreted from Listeria are rapidly degraded by the host cell

Listeria secretes a protein called ActA, which polymerizes cellular actin, allowing the bacterium to escape the host cell and infect neighboring cells (11). We chose to use an ActA-deficient form of Listeria for our in vitro kinetic studies because it is unable to polymerize host cell actin. This prevents Listeria from spreading from cell to cell during the course of the assay and, potentially, introducing greater variability in determining the kinetics of surface pMHC expression. By using this ActA-deficient form of Listeria, we were able to restrict the timing of the infection to the initial period of incubation with the attenuated Listeria.

To evaluate the role of protein half-life in pMHC production for recombinant proteins expressed from Listeria, we generated two attenuated ActA-deficient Listeria constructs expressing recombinant proteins with vastly different cellular stabilities (Fig. 1A). The first construct, termed AU-NP-S-FLAG (8), is a fusion protein consisting of the first 100 aa of the Listeria ActA protein, followed by a Ub moiety, full-length influenza NP, the model MHC class I K\textsuperscript{b} binding peptide SIINFEKL, and a 3× FLAG tag. ActA\textsubscript{1-100} contains a bacterial secretion signal that enables efficient entry of the fusion protein into the host cell but, importantly, lacks the actin binding domain (12). Inclusion of the Ub moiety allows for rapid and efficient liberation of NP-S-FLAG by resident Ub hydrolases in the host cell cytosol (13). Immediately following Ub is influenza NP, a model protein with a half-life measured in days (4). The inclusion of the SIINFEKL peptide allows for the precise quantification of surface pMHC production using the 25-D1.16 mAb, which specifically detects SIINFEKL peptide bound to the murine MHC class I K\textsuperscript{b} molecule (14). Lastly, the 3× FLAG tag facilitates detection of the fusion protein by Western blot analysis. Moreover, including a motif at the C terminus of the SIINFEKL moiety ensures that liberation of SIINFEKL is dependent on the activity of host cell proteasomes.

Except for the addition of an arginine at the N terminus of the NP moiety, AU-R-NP-S-FLAG is identical to AU-NP-S-FLAG. This single amino acid addition is used to generate a version of the NP-S-FLAG protein with a cellular half-life of ~10 min compared with days for the original NP-S-FLAG construct. Arginine residues at the N terminus of eukaryotic proteins act as N-end rule degrons, targeting the proteins for rapid degradation in a proteasome-dependent manner (1). Upon entry of the recombinant protein into the host cell, eukaryotic Ub hydrolases remove the N-terminal ActA-Ub moiety, exposing the N-terminal arginine of the recombinant R-NP-S-FLAG protein.

Because both proteins are expressed using the pPL2 integration vector, only one copy of each gene is expressed per bacterium (15). Importantly, this helps reduce, if not eliminate, the variability in the amount of protein secreted from different recombinant strains of Listeria. We established that both the AU-NP-S-FLAG and AU-R-NP-S-FLAG proteins were synthesized and secreted by Listeria at the same rate by inoculating eukaryotic cell culture medium with Listeria expressing either construct and then measuring protein levels in the growth medium at several time points post-inoculation. Eukaryotic growth medium (IMDM) is used for these in vitro secretion assays, as it initiates translation of proteins under the control of virulence promoters, including the ActA promoter used for the constructs in this study (12, 16). As shown in Fig. 1B, AU-NP-S-FLAG and AU-R-NP-S-FLAG are secreted at nearly identical rates in a cell-free system.

To determine whether AU-R-NP-S-FLAG is secreted in infected host cells at the same rate as AU-NP-S-FLAG, we treated the macrophage-like cell line BMA3 with 5 μM irreversible proteasome inhibitor epoxomicin (8) to prevent degradation of the R-NP-S-FLAG protein, then infected the BMA3 cells for 3 h with Listeria expressing either AU-NP-S-FLAG or AU-R-NP-S-FLAG. Infected cells were lysed and protein levels determined by Western blot analysis (Fig. 1C), confirming that AU-NP-S-FLAG and AU-R-NP-S-FLAG are secreted in Listeria-infected BMA3 cells at comparable rates. Importantly, in cells with functional proteasomes, R-NP-S-FLAG exhibits a significantly higher rate (p < 0.05) of degradation than does NP-S-FLAG, demonstrating it behaves as an N-end rule substrate comparable to those generated from host cell ribosomes.
Generation of pMHC from recombinant Listeria-derived NP is independent of the cellular half-life of the protein.

We previously showed that proteins synthesized by host cell ribosomes, such as those encoded by rVV, generate pMHC at a rate proportional to the amount of protein degraded (4). We confirmed this correlation between protein degradation and pMHC generation for endogenously synthesized proteins in BMA3 cells by infecting with rVV expressing stable and rapidly degraded forms of NP, comparable to those expressed from our recombinant Listeria (Fig. 2B). Importantly, note that surface pMHC values were measured only in live, infected cells. In both the Listeria and rVV systems used for these experiments, we begin to see significant levels of cell death by 5–6 h postinfection. Therefore, to minimize the potential impact of cell death on our pMHC measurements, we limited all kinetic analyses to the first 5 h of infection.

To determine whether protein stability plays a role in pMHC generation from secreted Listeria proteins, we infected BMA3 cells with Listeria expressing the stable AU-NP-S-FLAG or rapidly degraded AU-R-NP-S-FLAG forms of recombinant protein. Strikingly, both recombinant proteins generated surface Kβ-SIINFEKL with similar kinetics, irrespective of the cellular half-life of the protein from which they were generated (Fig. 2A). To ensure that this apparent lack of correlation between protein half-life and pMHC generation was not limited to the BMA3 cell line, we infected primary BMMΦs (Fig. 2C) and BMDCs (Fig. 2D) from C57BL/6 mice with either the AU-NP-S-FLAG– or AU-R-NP-S-FLAG–expressing Listeria constructs. Again, we found the kinetics of surface Kβ-SIINFEKL was completely independent of the cellular half-life of the protein from which they were derived.

It is possible that the lack of correlation between the amount of protein degraded and surface pMHC generated is specific for the recombinant protein construct used for these assays. To address this, we replaced the influenza NP portion of the recombinant protein with the N-terminal domain of the influenza PA protein (PA1–256), generating stable AU-PA-S-FLAG and rapidly degraded AU-R-PA-S-FLAG versions of the protein (Fig. 3A). We limited this construct to the first domain of PA, as we have found that increasing the size of a secreted Listeria protein can significantly reduce protein expression levels (8). As with the NP constructs, we confirmed that the two recombinant PA proteins were secreted at the same rate and that the N-terminal arginine acted as a destabilizing N-end rule degron (Fig. 3B). We then measured the kinetics of surface Kβ-SIINFEKL production in BMA3 cells (Fig. 3C), BMMDΦs (Fig. 3D), and BMDCs (Fig. 3E). In all cases, the rate of surface Kβ-SIINFEKL production from the stable and rapidly degraded recombinant proteins was equal and, importantly, independent of the cellular stability of the secreted recombinant protein.

Although we demonstrated that degradation of the N-end rule R-NP and R-PA degrons expressed from Listeria was dependent on proteasomal activity in infected cells (Figs. 1C, 3B), we wanted to determine if pMHC generation from Listeria-derived proteins followed the classical, proteasome-dependent MHC class I Ag processing pathway. We therefore infected BMA3 cells with AU-NP-S-FLAG (Fig. 4A), AU-R-NP-S-FLAG (Fig. 4B), AU-PA-S-FLAG (Fig. 4C), or AU-R-PA-S-FLAG (Fig. 4D). At 180 min postinfection, each sample was split and treated with either 5 μM epoxomicin or DMSO vehicle, and surface Kβ-SIINFEKL levels were measured for an additional 2 h. For each construct, surface Kβ-SIINFEKL expression was completely abrogated by the addition of the proteasome inhibitor, but unaffected by the DMSO vehicle. Importantly, the 20–40 min delay between treatment with epoxomicin and cessation of pMHC presentation matches the time delay seen in our previous findings for rVV-derived recombinant proteins (4). In addition, we infected BMMΦs from TAP−/− mice with our recombinant Listeria constructs. The complete absence of surface Kβ-SIINFEKL expression in these cells confirmed that Ag presentation was TAP dependent (data not shown). We also treated infected BMA3 cells with brefeldin A, which completely abrogated

![Figure 2](http://www.jimmunol.org/)
surface $K_b$-SIINFEKL expression, to demonstrate that presentation of $K_b$-SIINFEKL was dependent on Golgi transport (data not shown).

It is possible that \textit{Listeria} infection causes global changes in the Ag processing pathway that affects pMHC generation independent of the origin of the protein degraded. To address this possibility, we coinjected BMA3 cells with \textit{Listeria} and rVV, with only one of the vectors acting as a source of peptide, and determined the kinetics of $K_b$-SIINFEKL surface expression. In the first case, we infected BMA3 cells with rVV expressing NP-S-eGFP as the source of the SIINFEKL peptide and \textit{Listeria} expressing a nonsecreted form of the red fluorescent mCherry protein (Fig. 5A). In the reciprocal experiment, BMA3 cells were infected with \textit{Listeria} expressing a nonsecreted form of mCherry and AU-NP-S-FLAG as the source of SIINFEKL and rVV expressing only the yellow fluorescent Venus protein (Fig. 5B). Expressing red and green or yellow fluorescent proteins from \textit{Listeria} and rVV, respectively, allowed us to evaluate $K_b$-SIINFEKL levels only on those cells coinfected with both vectors. In both cases, the kinetics of presentation were determined by the vector from which the recombinant protein was expressed, and, importantly, was independent of coinfection with the complementary vector.

\textit{The efficiency of pMHC generation from recombinant proteins secreted from \textit{Listeria} is higher than for comparable endogenously synthesized proteins}

In light of the highly disparate efficiencies measured for pMHC generation from rVV and native \textit{Listeria} proteins (4, 5, 7, 17), we sought to determine the efficiency of pMHC generation from recombinant NP expressed from \textit{Listeria}. However, to calculate the efficiency of pMHC generation using the method employed for our rVV studies requires that there be a proportional relationship between the amount of protein degraded and the amount of surface pMHC generated. Because pMHC generation from the secreted \textit{Listeria}-derived proteins in these studies proved to be independent of the amount of protein degraded, we were unable to use this method to determine the efficiency of $K_b$-SIINFEKL production from \textit{Listeria}. Instead, we chose to establish what the minimal efficiency of surface $K_b$-SIINFEKL production from \textit{Listeria} would be if every recombinant protein secreted into the host cell were degraded and available as a source of MHC class I binding peptide. To accomplish this, we compared recombinant protein levels and surface $K_b$-SIINFEKL production from both rVV and \textit{Listeria}-expressed proteins, we re-
stricted our measurements to time points from 180 to 240 min postinfection, when pMHC generation was linear for both vectors. This approach is consistent with the method we used to determine processing efficiency in earlier experiments using proteins expressed from rVV (4). We found that there was little variation in the ratio of surface Kb-SIINFEKL generated from rVV- and Listeria-expressed proteins for each of two individual experiments (Table I). Using these measurements of surface pMHC complexes, we estimated that Kb-SIINFEKL generated from rVV-derived NP-S-eGFP was ∼2.8-fold greater than surface Kb-SIINFEKL levels generated from Listeria-derived AU-NP-S-FLAG. We then used lysates of the epoxomicin-treated cells to measure the levels of total protein synthesized in rVV- and Listeria-infected cells at 3 h postinfection (Fig. 6B). We have previously shown that processing and presentation of peptides derived from recombinant proteins expressed from both rVV and Listeria requires ∼40–50 min (4, 8). Therefore, the ratio of protein levels measured at 3 h postinfection corresponds to the ratio of pMHC levels measured at 180–240 min postinfection. We compared each lysate to an NP protein standard to determine that the concentration of NP-S-eGFP in rVV-infected cells was ∼54-fold higher than the concentration of AU-NP-S-FLAG in Listeria-infected cells (Table II).

The efficiency of Kb-SIINFEKL production from recombinant Listeria-derived protein was calculated by measuring the total recombinant protein, either synthesized in rVV-infected cells or secreted by Listeria into the cytosol of infected cells, that is necessary to generate equivalent levels of surface Kb-SIINFEKL expression in infected cells. Because the efficiency of Kb-SIINFEKL production from rVV-derived NP-S-eGFP is known (∼1 surface pMHC per 3000 protein molecules degraded) (4), we can use this difference in efficiency to arrive at a minimal processing efficiency for secreted recombinant Listeria proteins. By dividing the efficiency for rVV-infected cells (∼1 in 3000) by 19 we can calculate that the efficiency of Kb-SIINFEKL generation in Listeria-infected cells would be 1 surface complex for every 160 AU-NP-S-FLAG molecules entering the host cell. Importantly, note that our calculations reflect the minimum efficiency possible in our experimental system, as it is based on the degradation of every AU-NP-S-FLAG molecule secreted by the Listeria into the host cell cytosol. However, we know that only a fraction of the total AU-NP-S-FLAG synthesized is degraded. Therefore, the efficiency of pMHC generation must be significantly greater than 1 pMHC per 160 protein molecules degraded, and may well be within the range calculated by Pamer and colleagues (5, 7) for native Listeria proteins (1 pMHC per 3.5–35 proteins degraded).

**Discussion**

To date, there has been only one report of MHC class I Ag presentation occurring independently of the degradation rate of the protein from which the peptides presented are derived. In this study from Shastri and colleagues (18), cells were transfected with DNA encoding fusion proteins containing an N-terminal Ub moiety followed by recombinant proteins expressed either as stable proteins or N-end rule degrons. The one feature this study has in common with our own experimental approach is the expression of protein constructs containing Ub-protein motifs. Although it is conceivable that expression of these recombinant proteins as Ub-
protein chimeras may target the protein to a high efficiency processing pathway, the use of these motifs in rVV expression systems suggests otherwise. When Ub-R-NP-S-eGFP is expressed from rVV, it increases surface pMHC ∼3-fold relative to presentation from NP-S-eGFP, which corresponds directly with the difference in the amount of protein degraded for these two constructs (4). If the Ub-protein motif were responsible for targeting protein to the high efficiency pathway, then pMHC generation from Ub-R-NP-S-eGFP should be significantly greater than the ∼3-fold difference from NP-S-eGFP. As such, these data strongly suggest that it is expression from Listeria that is the determining factor in the targeting of recombinant protein to the high-efficiency processing pathway.

It is possible that the observed difference in processing efficiency for proteins expressed from rVV and Listeria is the result of the significantly higher levels of recombinant protein synthesized in rVV vs. Listeria-infected cells. However, a study by Wherry et al. (19), in which vaccinia virus was engineered to express different levels of a recombinant NP-SIINFEKL protein construct, suggests this is not the case. In that study, the level of NP-SIINFEKL expression between the lowest and highest expressing rVV differed by 200-fold, a range that is 4-fold greater than the difference in protein expression levels between the rVV and Listeria constructs used for the efficiency studies we report in this article. Wherry et al. demonstrated a direct correlation between the level of protein synthesized and surface Kβ-SIINFEKL expression across the entire 200-fold range of protein expression tested. Importantly, note that the levels of Kβ-SIINFEKL expression tested in the vaccinia study by Wherry et al. were comparable to those in our study using a similar NP-SIINFEKL construct and extended to levels several fold lower than those measured in our Listeria system when vaccinia with lowered protein expression was evaluated.

Approximately 10-fold more AU-R-NP-S-FLAG and AU-R-PA-S-FLAG are degraded when expressed from Listeria than from the corresponding stable protein constructs. Because the rapidly degraded proteins generate similar levels of surface Kβ-SIINFEKL as do the stable forms, it is possible that pMHC is
generated from the rapidly degraded proteins with at least a 10-fold lower efficiency than from the stable proteins. Although it is possible that processing occurs with different efficiencies for proteins with different cellular half-lives, we do not think this is likely considering the nearly identical presentation kinetics we observe for stable and rapidly degraded forms of two distinct proteins, one of which localizes to the cytosol (PA) and the other to the nucleus (NP). Instead, we hypothesize that only a certain proportion of *Listeria*-derived proteins are able to access a high-efficiency pMHC-processing pathway, and that access to this pathway is independent of the cellular half-life of the protein. Importantly, high-efficiency processing occurs almost immediately upon secretion of protein substrate by the *Listeria* into the host cell cytosol. Any *Listeria*-derived proteins that do not enter this high-efficiency pathway enter the cellular pool of protein and are subsequently processed at an efficiency low enough that it will not contribute significantly to surface pMHC levels. A recently published study by Grauling-Halama et al. (20) supports our findings. In that study, the authors use regression analysis of previously published data from Pamer et al. (19) to demonstrate a tight linkage between *Listeria* protein synthesis and pMHC generation. Although they do not directly measure production of pMHC complexes in *Listeria*-infected cells, their analysis of the Pamer groups data are completely consistent with our observations.

Although it is possible that this high-efficiency pathway is confined to proteins expressed from *Listeria*, results from our original rVV efficiency study (4), as well as more recent data from Dolan et al. (3), suggest otherwise. In the rVV study, we observed higher presentation efficiency for peptides derived from a cellular form of the NP-S-eGFP protein containing a destabilizing KEKE motif. This suggested to us that presentation efficiency may be higher for proteins that enter the cellular pool when compared with those processed in a near cotranslational manner, such as endogenously synthesized N-end rule degrons or proteins processed as defective ribosomal products (DRiPs) (21, 22). The study by Dolan et al. used biochemical techniques to distinguish between pMHC generated from proteins that have entered the cellular pool and pMHC generated from newly synthesized DRiPs. In that study, >50% of all pMHCs were generated from a very small fraction of nascent, endogenously synthesized proteins, which are an inefficient source of peptides for pMHC presentation and that pMHC generated from newly synthesized DRiPs are presented with at least a 6.5-fold greater efficiency than are "retirees."

The results we present in this study are consistent with those reported by Dolan et al. with one important difference. Although we observed the same high-efficiency processing of a small fraction of total protein synthesized, the source of these proteins cannot, by definition, be DRiPs. *Listeria* proteins are synthesized by bacterial ribosomes and cannot access the Ag processing machinery of the host cell until secreted by the bacterium. Therefore, entry of *Listeria*-derived proteins to a high-efficiency processing pathway cannot be the result of a direct association between the biosynthetic machinery and the Ag processing pathway of the host cell, as has been suggested for DRiPs (3, 23, 24). Rather, our results demonstrate that the high-efficiency processing pathway is accessible to both endogenously synthesized and exogenously derived proteins. Importantly, this suggests that the high-efficiency processing pathway is accessible to multiple sources of antigenic protein, which may include proteins entering the cross-presentation pathway of professional APCs. The specific factors that target a protein for processing by the high-efficiency pathway remain to be elucidated. However, demonstrating that virtually all pMHC generation from *Listeria*-derived proteins follows this pathway should greatly facilitate our ability to define differences in the MHC class I processing pathway that result in high- and low-efficiency presentation of specific antigenic proteins.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**


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**Table I.** Surface K<sup>b</sup>-SIINFEKL complexes used for calculating the efficiency of K<sup>b</sup>-SIINFEKL generation from recombinant *Listeria* proteins

<table>
<thead>
<tr>
<th>Experiment 1</th>
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<td>200</td>
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<tr>
<td>7,189</td>
<td>2171</td>
<td>4.0</td>
<td>3.5</td>
</tr>
</tbody>
</table>

**Table II.** Molecules of rVV-derived NP-S-eGFP or *Listeria*-derived AU-NP-S-FLAG protein per cell at 3 h postinfection

<table>
<thead>
<tr>
<th>Experiment</th>
<th>rVV NP-S-eGFP</th>
<th>Listeria NP-S-FLAG</th>
<th>Ratio rVV/L monocytogenes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td>$8.49 \times 10^6$</td>
<td>$2.44 \times 10^6$</td>
<td>34.8</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>$9.12 \times 10^5$</td>
<td>$1.25 \times 10^6$</td>
<td>73.0</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td>54</td>
</tr>
</tbody>
</table>


