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Cross-Presentation of Listeria-Derived CD8 T Cell Epitopes Requires Unstable Bacterial Translation Products

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Presentation of bacteria-derived CD8 T cell epitopes by dendritic cells (DC) requires either their direct infection or that DC acquire and cross-present Ags from other infected cells. We found that cross-presentation of Listeria monocytogenes-derived CD8 T cell epitopes was much stronger than direct Ag presentation by infected murine DC. Cross-presentation of Listeria-derived CD8 T cell epitopes showed unique physiological requirements. It was dependent upon the delivery of unstable bacterial translation products by infected, but still viable, Ag donor cells. Cross-presentation was enhanced both when unstable translation products in infected Ag donor cells were protected from proteasomal degradation and when the production of misfolded bacterial proteins was increased. The requirement of unstable translation products for cross-presentation may represent a novel pathway that functions to focus the CD8 T cell response toward epitopes derived from newly synthesized proteins. The Journal of Immunology, 2004, 173: 5644–5651.

The control of viral and intracellular bacterial pathogens requires the rapid recognition of infected cells by CD8 T cells that recognize peptides of eight to 11 aa presented on the cell surface by self-MHC class I molecules. Infected cells generate antigenic peptides after proteasome-mediated intracellular degradation of pathogen-derived proteins. In bone marrow-derived professional APC, an alternative MHC class I Ag presentation pathway is also present; dendritic cells (DC) and macrophages have the exclusive ability to cross-present Ags taken up from other infected cells (1, 2). For the APC, the advantage of cross-presentation is that it is not infected, and therefore, it is protected from any cytopathic effect exerted by the intracellular pathogen. The mechanisms involved in cross-presentation are not fully understood, and a number of alternative models exist. Antigenic material is taken up either in the form of peptides bound to heat shock proteins (3) or in the form of complete proteins that subsequently are processed and presented in the classical cytoplasmic MHC class I presentation pathway (4) or by an alternative endosomal pathway (5).

In BALB/c mice, Listeria monocytogenes infection stimulates a strong protective CD8 T cell response against p60217–225 and aa 91–99 of listeriolysin O (LLO) (6). The induction of the L. mono-

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4 Abbreviations used in this paper: DC, dendritic cell; DRiPs, defective ribosomal product; LLO, listeriolysin O; p.i., postinfection; wt, wild type.

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Materials and Methods

Mice, cell lines, and, bacteria

Female BALB/cOlaHsd (H-2b) mice were purchased (Janvier, Le Genest St. Isle, France), kept under conventional conditions, and used at 8–10 wk of age. Macrophage-like IC21 cells (H-2b) were used as Ag donor cells and were kept in DMEM supplemented with 10% FCS without antibiotics. P388D1 (H-2b) cells were used as APC for the analysis of direct Ag presentation by infected cells.

Listeria monocytogenes serovar 1/2a EGD and L. monocytogenes SaintA (11) were grown in brain heart infusion broth (BD Biosciences, Heidelberg,
Germany) and were used in the logarithmic growth phase. The bacterial concentration was estimated from the OD at 600 nm.

Construction of recombinant vaccinia virus

The open reading frame encoding \textit{L. monocytogenes} p60 was amplified by PCR using primers p60 forward (5'-ggg ttc tag acc acc atc gta gta gtc gaa gct ggt-3') and p60 reverse (5-cgg agg atc cgt ata cgc gaa age caa ct-3') and plasmid pSK5 (12) (provided by Dr. I. Gentschev, Biozentrum, University Würzburg, Würzburg, Germany) as a template and was subcloned into pUC19. An XbaI site in front of the ATG start codon was converted to a BamHI site by insertion of a self-complementary oligonucleotide (5'-ctg gca gca gtc gaa gct ggt tcg-3'), then the p60 open reading frame was transferred as a 1.5-kbp BamHI fragment into the vaccinia recombinant vector pC543 (13). Construction of the recombinant vaccinia virus was performed using the vaccinia virus strain Copenhagen and its temperature-sensitive mutant ts7 according to standard procedures as described previously (13).

\textbf{T cell lines}

CD8 T cell lines specific for p60 217–225 and LLO 91–99 (both K\(^t\)-restricted) and a CD4 T cell line directed against the H-2A\(^k\)-restricted epitope p60 312 were established and propagated as described previously (10, 14).

\textbf{Bone marrow-derived DC}

DC were obtained from GM-CSF-supplemented (Tebu-Bio, Offenbach, Germany) bone marrow cultures as described previously (10). DC were used after 5 days of culture. In experiments in which optimal purity of DC was required, DC were further purified with anti-CD11c-coated magnetic beads (Miltenyi Biotec). In contrast to directly infected cells, cross-presenting DC take up from Ag donor cells, was prevented by culturing DC in medium containing 10 \mu m L-canavanine or puromycin, the coculture of DC and infected Ag donor cells was set up as described above. After 7 h, cytoplasmic preparations of cells were prepared, and intracellular bacteria were stained. After fixation of cells with 2% paraformaldehyde and Triton X-100 treatment (0.05% for 30 s), intracellular bacteria were stained with a polyclonal rabbit anti-Listeria type I and 4 antiserum (BD Biosciences) and a donkey anti-rabbit Cy5-conjugated IgG Fab\(^2\) (Jackson ImmunoResearch Laboratories, West Grove, PA). Cellular actin filaments were visualized with Alexa Fluor 488 phalloidin (Molecular Probes, Leiden, The Netherlands). All samples were mounted with PROLONG mounting medium (Molecular Probes). Images were acquired using a conventional DMRE fluorescence microscope (filter blocks N2.1 and Y5 for green and far red fluorescence, respectively; Leica Microsystems, Wetzlar, Germany).

\textbf{TUNEL assay}

The degradation of nuclear DNA in apoptotic cells was detected using a commercial APO-BrdU TUNEL assay kit (Molecular Probes). Briefly, IC21 cells were grown in six-well plates and were harvested 9 h after infection. As a positive control, apoptosis of IC21 cells was induced by UV irradiation using two OSRAM HNS 15-W OFR bulbs (254 nm) for 10 min with a distance of 4 cm between bulb and cells (0.04 \mu W/cm\(^2\)). Approximately 1–2 \times 10\(^6\) cells were fixed with 1% paraformaldehyde in PBS and permeabilized for 30 min in 70% ethanol. After washing, break sites in the DNA were labeled with 5-bromo-dUTP and detected by a fluorescein-conjugated donkey anti-mouse IgG secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Nuclear DNA was visualized with DAPI (Molecular Probes, Leiden, The Netherlands).

\textbf{Results}

Cross-presentation of \textit{L. monocytogenes}-derived CD8 T cell epitopes requires bacterial replication in the Ag donor cell

In contrast to directly infected cells, cross-presenting DC take up Ags from infected cells without being infected themselves. Because direct Ag presentation requires bacterial protein synthesis (8, 9), we examined whether cross-presentation of \textit{L. monocytogenes}-derived Ags has different requirements. DC poorly presented p60- or LLO-derived CD8 T cell epitopes after infection with \textit{L. monocytogenes}, but much stronger presentation occurred after incubation of DC (H-2\(^b\)) with \textit{L. monocytogenes}-infected MHC-disparate IC21 cells (H-2\(^b\); Fig. 1). After infection at a multiplicity of infection of 10 for 1 h, replicating bacteria were detected in 20–30% of cells (data not shown). Bacterial replication was obviously by the presence of groups of multiple intracellular bacteria (Fig. 2A). To prevent cross-infection by spreading of bacteria from infected cells to DC, we used Ag donor cells infected with the non-spreading \textit{L. monocytogenes} \(\Delta\text{actA}\) mutant (20, 21). No cell-to-cell spread of bacteria occurred, as demonstrated by mixing of infected unlabelled Ag donor cells with covalently labeled DC (Fig. 2B). Staining of \textit{L. monocytogenes} with a polyclonal rabbit antisem detected bacteria in the unlabelled IC21 cells, but not in fluorescently labeled DC that were added to the infected IC21 cells after killing and removal of extracellular \textit{L. monocytogenes}. To detect whole bacteria or bacterial fragments in DC with maximum sensitivity, the red \textit{Listeria}-specific fluorescence was deliberately overexposed, which resulted in the visual merging of individual bacteria in strongly infected IC21 cells (Fig. 2B, lower right quadrant). The strength of cross-presentation of p60 217–225 by DC was similar with Ag donor cells infected with \textit{L. monocytogenes} wt or \textit{L. monocytogenes} \(\Delta\text{actA}\), respectively (Fig. 3A, upper panel). Remarkably, no cross-presentation of p60 217–225 occurred if DC and...
Ag donor cells were mixed in the presence of clinafloxacine and gentamicin that kill intra- and extracellular L. monocytogenes, whereas cross-presentation of the CD4 epitope p60<sub>217-225</sub> (Fig. 3A, lower panel) was not inhibited in the presence of clinafloxacine and gentamicin. MHC class I-restricted cross-presentation was also abrogated if Ag donor cells were lysed by repeated freeze-thaw cycles (Fig. 3A). Similar to p60<sub>217-225</sub>, cross-presentation of LLO<sub>91-99</sub> was inhibited if bacterial protein synthesis was suppressed by antibiotics (Fig. 3B). The failure to achieve cross-presentation if bacterial protein synthesis was inhibited or if Ags were delivered by cells killed by freeze-thawing could not be overcome by prolonged infection of Ag donor cells or by infection with an enhanced number of bacteria per cell (data not shown). A kinetic analysis revealed the strongest cross-presentation by DC after 7 h of coculture with Ag donor cells (Fig. 3C). Cross-presentation was significantly lower if the period was much shorter (3 h) or longer (16 h).

To exclude a possible bias toward Ag donor cells harboring viable Listeria due to the accumulation of additional proteins during the coculture of DC, we tested cross-presentation under conditions where the total time allowed for infection, and thus the total amount of bacterial Ags accumulated in infected Ag donor cells, were kept constant (Fig. 4). Infected Ag donor cells were cocultured with DC 30 min before, 1 h before, immediately after, or 30 min after blocking of protein synthesis by addition of clinafloxacine. Under these conditions, a part of the coculture of Ag donor cells and DC occurred in the presence of gentamicin only (Fig. 4; coculture starting 4 or 4.5 h p.i.) or clinafloxacine was present during the whole coculture of Ag donor cells and DC (Fig. 4; coculture starting at 5 or 5.5 h p.i.). In all groups the total time allowed for bacterial replication was kept constant (5 h) as was the duration of the coculture of Ag donor cells and DC (5 h). Under these experimental conditions, cross-presentation correlated clearly with the presence of viable bacteria in the Ag donor cells during the coculture with DC (Fig. 4B). If bacterial protein synthesis was blocked by clinafloxacine before the addition of DC, cross-presentation of p60<sub>217-225</sub> and LLO<sub>91-99</sub> was significantly reduced. These data indicate that efficient cross-presentation requires a form of Ag that is provided only in cells infected with viable, replicating bacteria.

It has been reported that in certain experimental systems, apoptosis of the Ag donor cells (22) or immune complexes (23) are required for cross-presentation. A TUNEL assay with L. monocytogenes ΔactA-infected IC21 cells revealed no DNA fragmentation in infected cells, indicating that bacteria did not induce apoptosis in infected cells (Fig. 5A). The viability of infected cells 9 h p.i. was still ~80%. If bacterial protein synthesis was inhibited by clinafloxacine, neither UV radiation of infected Ag donor cells (Fig. 5B) nor serum from L. monocytogenes-immune mice (data not shown) restored cross-presentation of p60<sub>217-225</sub> or LLO<sub>91-99</sub>.
engulf Ag donor cells and actively take up antigenic material. As shown in Fig. 2B, neither whole bacteria nor large bacterial fragments were transferred from infected Ag donor cells to DC during cross-presentation. However, because bacteria rapidly multiplied in DC (Fig. 2A), even a low percentage of infected DC might be sufficient to stimulate T cells. To prevent the principal possibility of bacterial infection and intracellular protein expression, DC were pretreated with azithromycin, a highly hydrophobic macrolid antibiotic that accumulates intracellularly and prevents growth of L. monocytogenes-infected cells (18). Intracellular L. monocytogenes wt did not show actin polymerization after infection of azithromycin-treated P388 cells (data not shown), and azithromycin-treated P388 cells infected with L. monocytogenes did not present L. monocytogenes-derived CD8 T cell epitopes even if cells were infected 4 h after azithromycin was washed out (Fig. 6A). A possible interference of azithromycin or clinafloxacin with the cytosolic Ag presentation pathway was excluded by a control experiment with APC infected with a recombinant vaccinia virus expressing p60 (Fig. 6B). Cross-presentation of L. monocytogenes-derived Ags by azithromycin-loaded DC showed similar results as cross-presentation by untreated DC (Fig. 6C), excluding bacterial infection and intracellular protein synthesis as the source of Ags in cross-presenting DC.

**Regulation of costimulatory molecules on DC during cross-presentation**

Cross-presentation requires uptake of Ags and activation of DC. Uptake of Ags by DC is followed by the maturation of DC, down-regulation of phagocytic activity, and concomitant up-regulation of costimulatory molecules.
of costimulatory molecules (1). Differences between Ag donor cells that harbor dead or viable bacteria, respectively, in their ability to activate DC and trigger the expression of costimulatory molecules could explain why cross-presentation is blocked by antibacterial treatment of Ag donor cells. However, cytofluorometric analysis of DC from cross-presentation cultures revealed similar strong up-regulation of CD80 and CD86 on DC independent of bacterial replication in Ag donor cells (Fig. 7), indicating that bacterial replication in Ag donor cells was not required for the maturation of DC.

Processing of cross-presented Ags

During cross-presentation, Ags could be transferred from the Ag donor cell to DC either unprocessed, partially processed, or finally processed. To learn more about the state of the transferred Ags, we tested the effect of the proteasome inhibitor epoxomycin on cross-presentation (Fig. 8A). Epoxomycin treatment of DC suppressed cross-presentation, indicating that the Ags taken up by DC are not finally processed. Quantitatively, the inhibitory effect of epoxomycin pretreatment of DC was on the same order of magnitude as the inhibition of cross-presentation by clinafloxacin (Fig. 8A). The strict requirement for proteasome-mediated Ag processing by DC also ruled out peptide regurgitation (5), a scenario that is also highly unlikely, because in our system the Ag donor cell and the APC did not share MHC class I molecules. Transfer of soluble material was also not observed in experiments in which DC and Ag donor cells were separated by a semipermeable membrane (2-µm pore diameter) that totally inhibited cross-presentation (data not shown). In contrast, epoxomycin treatment of Ag donor cells did not inhibit cross-presentation, indicating that the Ags transferred from the infected cells to DC did not require proteasomal preprocessing in the Ag donor cell (Fig. 8A).

Cross-presentation of L. monocytogenes-derived CD8 T cell epitopes was strictly inhibited by the blocking of bacterial protein synthesis during coculture with DC. This requirement is consistent with the Ag presentation model developed by Yewdell and colleagues that suggests that short-lived defective ribosomal products (DRiPs) are an important substrate for the cytosolic Ag presentation pathway (24). If unstable proteins are transferred from infected Ag donor cells to DC during cross-presentation, stabilization of these proteins should enable cross-presentation in the...
absence of bacterial protein biosynthesis. Therefore, a cross-presentation experiment with epoxomycin-treated, L. monocytogenes-infected Ag donor cells was performed. Although under conditions of continuous bacterial protein biosynthesis, epoxomycin did not influence the level of cross-presentation (Fig. 8A) in the presence of clinafloxacin, which inhibits bacterial protein synthesis, the protection of presumptive unstable p60 and LLO translation products by epoxomycin resulted in enhanced cross-presentation of p60_217–225 and LLO_91–99 (Fig. 8B).

The percentage of defective unstable proteins formed during protein biosynthesis can be increased by agents such as puromycin (25) or L-canavanine (26), which are incorporated into growing polypeptide chains and result in the generation of misfolded unstable proteins. Remarkably, the treatment of Ag donor cells with either 1 μg/ml puromycin or 15 mM L-canavanine resulted in a strong increase in cross-presentation of p60_217–225 and LLO_91–99 (Fig. 9). At these concentrations, neither puromycin nor L-canavanine interfered with intracellular or extracellular bacterial replication and protein synthesis, respectively. DC were isolated by immunomagnetic separation, stained, and subjected to cytofluorometric analysis. Similar results were obtained in two independent experiments.

Discussion
In a previous report we demonstrated that the MHC class I-restricted presentation of L. monocytogenes-derived proteins by DC requires cross-presentation of Ags taken up from infected Ag donor cells (10). In this report we extend these findings and demonstrate that MHC class I-restricted Ag presentation by DC similarly is much more effective if DC remain uninfected and cross-present antigenic material taken up from other infected cells. We also show that MHC class I- and MHC class II-restricted cross-presentation have different physiological requirements. MHC class I-restricted cross-presentation of L. monocytogenes-derived proteins specifically requires that an unstable form of bacterial protein be taken up form viable Ag donor cells, whereas MHC class II-restricted cross-presentation is dependent upon neither viable Ag donor cells nor newly synthesized bacterial proteins.

The induction of an L. monocytogenes-specific CD8 T cell response in mice requires the presence of DC (7). DC presenting L. monocytogenes-derived Ags could either be infected directly or present exogenous material by cross-presentation. Our in vitro data are clearly in favor of the cross-presentation model, because directly infected DC poorly presented LLO- or p60-derived CD8 T cell epitopes, whereas efficient presentation occurred if DC were cocultured with infected Ag donor cells. This is probably due to the apoptogenic effect of LLO on DC and lymphocytes (27, 28). By taking up Ags from other infected cells, DC and T cells avoid the detrimental effect of LLO. Because not all DC were infected, Ag from infected DC could be taken up and cross-presented by uninfected DC. However, we found that bacteria residing in dead or apoptotic cells were cross-presented weakly (see Figs. 3A and 5B), probably due to the rapid inhibition of bacterial protein synthesis after influx of extracellular gentamicin into dying cells. Second, the cell density in the direct infection experiments was also different. In cross-presentation cultures, a high cell density (1 × 10^6 DC in 1 ml of medium) ensured the direct cell-to-cell contact of Ag donor cells and DC.

Professional APC have the ability to present exogenous nonviable Ags in the context of MHC class I molecules (4, 5, 29, 30). In DC, cross-presentation occurs in an endoplasmic reticulum-phagosome fusion compartment in which the functions of protein processing and peptide loading are present in direct neighborhood (31, 32). It is not known whether this processing pathway is identical with the pathway involved in the cross-presentation of cell-bound...
Ags. Ags from a nonreplicating source do not reflect the physiological situation of cross-presentation in vivo, in which intracellular replication of the pathogen and uptake of Ags from infected cells by DC are most likely synchronous events. The cross-presentation pathway that we studied strictly required continuous protein synthesis in Ag donor cells. In this respect, the pathway engaged in cross-presentation of L. monocytogenes-derived CD8 T cell epitopes is clearly different from the pathway described in a number of cellular (29), viral (22, 33), and bacterial (34) cross-presentation models in which Ags were delivered by apoptotic or necrotic cells, and continuous protein biosynthesis was not required. This difference is also reflected by the observation that a relatively long time (7 h) was necessary to obtain optimal cross-presentation, whereas efficient cross-presentation of Ags from a nonreplicating source has been reported to occur after 1 h of coculture of DC and Ag donor cells (33).

Why does MHC class I-restricted cross-presentation of L. monocytogenes-derived antigenic peptides require continuous bacterial protein synthesis? In general, accumulated Listeria-derived proteins in Ag donor cells could be present in an insufficient amount or in a state that does not support cross-presentation. If bacteria double intracellularly every 60 min, it could be expected that after an extended growth period of, e.g., 5 h, ~30-fold more bacteria and bacterial proteins would be present in the infected cell. Three lines of evidence indicate that the cross-presentation pathway described in this report does not simply depend on the pure amount of protein that accumulated in Ag donor cells, but requires the presence of an unstable form of Ag. First, the cross-presentation of Ags from Ag donor cells harboring replicating bacteria was stronger compared with nonreplicating bacteria independent of the total Ag load of the Ag donor cells (see Fig. 4), and the failure to achieve cross-presentation could not be overcome by prolonged infection or by infection of Ag donor cells with an enhanced number of bacteria per cell. Second, after inhibition of bacterial protein synthesis in Ag donor cells, the inhibition of the proteasome by epoxomycin restored cross-presentation. This effect of epoxomycin can only be understood if it is assumed that besides the normal stable bacterial proteins (that in the case of p60 are degraded with a t₁/₂ of ~90 min) (8) unstable translation products with a shorter half-life are also generated. If bacteria and thus also the protein production rate grow exponentially, and new proteins decay with a t₁/₂ of 90 min, ~25% of the synthesized proteins would be degraded during the first 4 h of infection. Thus, if epoxomycin protects only this 25% of proteins, this would not significantly change the total load of bacterial proteins in infected cells. The third line of evidence is that the induction of DRiP formation in Ag donor cells enhanced cross-presentation. Puromycin (25, 35) and canavanine (26) are erroneously integrated into the growing polypeptide chain by the ribosome in place of normal amino acids and result in truncated and aberrantly folded nascent polypeptides that are rapidly degraded by the proteasome. These truncated nascent proteins are considered equivalent to naturally occurring DRiPs (15, 16) that in eukaryotic cells constitute ~30% of newly synthesized proteins (36). In contrast to the role of DRiPs during direct Ag presentation (24), DRiPs have not yet been reported to play a role as an Ag source during cross-presentation. Because most cross-presentation studies used noninfectious Ags to avoid cross-infection, there have been few chances to observe this specific requirement. Two studies show that virally infected viable cells can be effective Ag donors (37, 38). However, in these two experimental systems, Ags from apoptotic cells were also cross-presented, indicating that continuous viral protein synthesis was not an absolute requirement for cross-presentation. A possible pathway for the uptake of DRiPs from Ag donor cells would be DC nibbling, a process that involves scavenger receptor A and enables DC to take up Ags from viable cells (39, 40). The failure to detect bacteria or bacterial fragments in DC suggests that cross-presentation did not depend on cross-infection or the uptake of whole bacteria or large bacterial fragments by DC.

The current study focused on an in vitro Ag presentation system. Only the in vitro manipulation of Ag donor cells and DC allowed us to pinpoint the physiological requirements necessary for cross-presentation of L. monocytogenes-derived Ags. Preliminary in vivo tests revealed that immunization of mice with Ag donor cells harboring no viable bacteria failed to stimulate a strong LLO- or p60 Listeria-specific CD8 T cell response (J. Janda and G. Geginat, unpublished observations). However, the alternative situation, i.e., immunization with Ag donor cells that harbor viable bacteria that are strictly confined to their host cells, cannot accurately be tested in vivo. This is a principle problem if studying cross-presentation of infectious microorganism that could directly infect professional APC in vivo. It also must be noted that the requirement of bacterial protein synthesis for the induction of an L. monocytogenes-specific CD8 T cell is not absolute in vivo, as demonstrated by the antigenic potential of very high doses of heat-killed Listeriae (41, 42). In a recent report, Norbury et al. (43) showed that in vivo cross-presentation favors stable Ags. Similar to the results reported by Norbury et al. (43) and in contrast to the study by Serna et al. (37), we found that Ags transferred during cross-presentation do not require proteasome-mediated Ag processing in the Ag donor cells. Norbury et al. (43) immunized mice with vaccinia virus-infected cells that were UV-inactivated. Between UV irradiation and contact with DC in secondary lymphatic organs, unstable proteins might already be degraded, which might explain why stable Ags were required for cross-presentation in vivo.

Taken together, our data suggest the existence of a specific cross-presentation pathway that requires that during cross-presentation, unstable bacterial translation products be transferred from viable Ag donor cells to DC in which proteasomal Ag processing occurs. By this pathway, Ag presentation by DC that cross-present
Ags is focused on antigenic peptides derived from freshly synthesized proteins. In light of the short time required for intracellular multiplication of bacteria or viruses, this is an important advantage for the infected host, because only the prompt recognition and elimination of infected cells can prevent the spread of an intracellular infection.

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