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Cross-Presentation of *Listeria monocytogenes*-Derived CD4 T Cell Epitopes

Mojca Škoberne, Simone Schenk, Herbert Hof, and Gernot Geginat

*L. monocytogenes* mediates the evasion of *Listeria monocytogenes* from the phagolysosome into the cytoplasm of the host cell. The recognition of infected cells by CD4 T cells is thought to be limited by the evasion of bacteria from the phagolysosome and also by the direct LLO-mediated inhibition of CD4 T cell activation. To analyze the influence of these immunoevasive mechanisms on the antilisterial CD4 T cell response, the expansion of *L. monocytogenes*-specific CD4 and CD8 T cells was monitored in infected mice. It was found that expansion of *L. monocytogenes*-specific CD4 T cells occurred synchronously with CD8 T cell expansion. The analysis of Ag presentation by macrophages and dendritic cells isolated from spleens of infected mice revealed efficient presentation of *L. monocytogenes*-derived CD4 T cell epitopes that was not dependent on the actA-mediated intercellular spread of bacteria. The further in vitro Ag presentation analysis revealed that although *L. monocytogenes*-infected macrophages and dendritic cells were poor presenters of CD4 T cell epitopes, more efficient presentation occurred after cocultivation of noninfected dendritic cells or macrophages with infected cells. These data indicate that the suppressive effect of LLO on the antilisterial CD4 T cell response is maintained only in infected APC and support the hypothesis that cross-priming plays a role in the induction of the strong CD4 T cell response in *Listeria*-infected mice. *The Journal of Immunology*, 2002, 169: 1410–1418.

The most important virulence factor for the pathogenesis of infection with *Listeria monocytogenes* is the sulfhydryl-activated hemolysin, listeriolysin O (LLO), the product of the hly gene (reviewed in Ref. 1). Secretion of LLO allows *L. monocytogenes* to escape from the endosomal compartment and to enter the cytosol of the host cell where intracellular growth occurs (2). At this stage, secreted bacterial proteins are directly fed into the cytosolic Ag-processing and presentation pathway which ends with the presentation of bacteria-derived naturally processed antigenic peptides in the context of MHC class I molecules on the cell surface (3). Second to mediating the escape from the endosome, LLO functions as an immune modulator (4) and also is an important target Ag for *L. monocytogenes*-specific CD4 and CD8 T cells (5–8).

A consequence of the cytoplasmic location of *L. monocytogenes* is that secreted bacterial proteins do not have direct access to the endosomal Ag presentation pathway. Expression of LLO by *L. monocytogenes* results in diminished CD4 T cell recognition of LLO itself and the murine hydrolase p60 (9, 10). Detailed analysis revealed that this effect on MHC class II-restricted Ag presentation depends primarily on the LLO-mediated change of the intracellular location of bacteria, given that purified LLO also inhibits the ability of CD4 T cell lines to recognize APC loaded with model Ag as OVA (11). The mechanism by which LLO inhibits target cell recognition by CD4 T cells is still not fully understood (12).

Despite this immunoevasive function of LLO, *L. monocytogenes*-infected mice mount a CD4 T cell response against a number of LLO and p60-derived epitopes (8). In BALB/c mice, the *L. monocytogenes*-specific T cell response is dominated by CD8 T cells directed against LLO91–99. Only a minor part of *L. monocytogenes*-specific T cells is directed against the CD4 T cell epitopes LLO189–200, LLO216–227, LLO211–222, p60337–378, p60118–149, and p60300–311. The antigenic region of LLO189–200 is conserved in C57BL/6 mice, where CD4 T cells directed against the related epitope LLO190–201 constitute the immunodominant T cell population. Further H-2b-restricted CD4 T cell populations are directed against LLO318–329, p60177–188, and p60301–412.

The induction of a strong *L. monocytogenes*-specific CD4 T cell response in infected mice cells into questions the in vivo efficacy of the LLO-mediated inhibition of CD4 T cell recognition. To elucidate this paradoxical strong *L. monocytogenes*-specific CD4 T cell response, the MHC class II-restricted Ag presentation by professional APC isolated from infected mice was studied. For this purpose, we used an ex vivo Ag presentation assay (13, 14). The immunodominant CD4 T cell response correlated with strong presentation of *Listeria*-derived CD4 T cell epitopes by dendritic cells (DC). Further in vivo Ag analyses were performed to elucidate the presentation pathway that results in strong MHC class II-restricted presentation of *Listeria*-derived Ag by DC. In summary, our results indicate that DC that acquired *Listeria*-derived Ag by cross-presentation are much more efficient stimulators of CD4 T cells than infected DC, suggesting that the suppressive effect of LLO on the *L. monocytogenes*-specific CD4 T cell response is not maintained in APC that cross-present *Listeria*-derived Ags.

**Materials and Methods**

*Mice, bacteria, and infection of mice*

Female BALB/c-OlaHsd (H-2d), C57BL/6OlaHsd (H-2b), CBA/CaOlalHsd (H-2b), and C57BL/6Rj × BALB/cRj F1 (B6CF1, (H-2b × H-2d)) mice were...
purchased (Harlan-Winkelmann, Borchen, Germany; Janvier, Le Geneste St. Isle, France), kept under conventional conditions, and used at 8–10 wk of age. Mice were infected with L. monocytogenes sororov 1/2a EGD, L. monocytogenes ΔactA (15), or L. monocytogenes Δlyt (16), in 0.2 ml of PBS i.v. as indicated. Bacteria used for infection were in the logarithmic growth phase. The bacterial concentration was estimated from the OD600. Heat-killed Listeria (HKL) were inactivated 2 h at 80°C; washed twice in PBS, and stored at −80°C.

CD8 and CD8 T cell lines

CD8 T cell lines specific for p60(17–226), p60(649–457), p60(665–686), and LLO0-99, and CD4 T cells against the H-2b-restricted CD4 T cell epitope p60(67–71) were derived from spleens of L. monocytogenes-infected BALB/c mice. CD4 T cell lines specific for the H-2b-restricted CD4 T cell epitope p60(98–100) were isolated from spleens 14 days after i.v. infection of C57BL/6 mice with 1 × 10⁶ CFU L. monocytogenes. A H-2L₄-restricted murine CMV, pp89(168–176)-specific CD8 T cell line (17) was kindly provided by R. Holtappels (University of Mainz, Mainz, Germany). All CD8 T cell lines were propagated by repeated restimulation with PB15 cells transduced with the human B7-1 gene (P815/B7) (18) in the presence of the appropriate synthetic peptide in medium supplemented with IL-2 as described previously (19). All CD4 T cell lines were repeatedly restimulated with syngeneic mitomycin C-inactivated splenocytes as APC in the presence of 10⁻⁶ M peptide. The T cell culture medium was a modification of Eagle’s medium (Invitrogen, Karlsruhe, Germany) supplemented with glutamine, penicillin, streptomycin, 10% FCS, 100 U/ml recombinant murine IL-2 (R&D Systems, Wiesbaden, Germany), and 2 × 10⁻⁶ M mercaptoethanol.

Immunomagnetic isolation of macrophages and DC

Cells expressing CD11b (20) or CD11c (21) were isolated by immunomagnetic cell sorting from spleen cell suspensions. Cells were selected with paramagnetic microbeads conjugated to monocolonal hamster anti-mouse-CD11b (clone M1/70.15.11.5; Miltenyi Biotec, Bergisch Gladbach, Germany) and anti-mouse-CD11c Abs (clone N418; Miltenyi Biotec), respectively. Spleens were injected with 500 μl of 1 mg/ml solution of colloidal graphite (50 μm in diameter; Mahllmann, Wiesbaden, Germany) in HBSS. Subsequently, spleens were cut into small pieces and incubated for 30 min at 37°C under 7.5% CO₂ in the collagenase D buffer. Cells were collected by centrifugation and separated twice on positive selection columns (Miltenyi Biotec) following the standard positive selection protocol provided by the manufacturer. For each experiment, spleens from three mice were pooled. At the end of the positive selection procedure, between 0.5 × 10⁶ positive cells were obtained per 1 × 10⁶ spleen cells. Aliquots of the selected cells were stained with FITC-labeled rat anti-mouse-CD11b IgG₂b mAb (clone M1/70; BD Biosciences, Heidelberg, Germany), rat IgG₁ isotype control mAb (A95-1; BD Biosciences), hamster anti-mouse CD11c IgG₂a mAb (clone HL3; BD Biosciences), and hamster IgG₁ isotype control mAb (clone G235-2356; BD Biosciences), respectively, and subjected to FACS analysis (data not shown). Dead cells were excluded from analysis by staining with propidium iodide. Cells selected with anti-CD11c microbeads were generally >80% pure DC. Cells selected with anti-CD11b microbeads were always CD11c-negative. A differential cell count revealed that macrophage-like mononuclear cells selected by anti-CD11b microbeads from spleens of infected mice were contaminated by ~30% polymorphonuclear granulocytes (18). Expression of MHC class II (PE-labeled rat anti-mouse-IA, IE, IgG₂b mAb, clone M5/114.15.2; BD Biosciences) and costimulatory molecules CD40 (PE-labeled rat anti-mouse CD40 IgG₂a, clone 3/23; BD Biosciences), CD80 (PE-labeled hamster anti-mouse-CD80-PE (B7-1) IgG₂, clone 16-10A1; BD Biosciences), and CD86 (PE-labeled rat anti-mouse CD86-PE (B7-2), IgG₂a mAb, clone GL1; BD Biosciences) was measured after staining with the indicated specific and PE-labeled isotype-matched control mAb (BD Biosciences).

Bone marrow cultures

Alternatively to ex vivo separation, macrophages and DC were obtained from bone marrow cultures (22, 23) in DMEM (Invitrogen) supplemented with 10% FCS and 10 mg/ml GM-CSF (R&D Systems). DC cultures were seeded in a density of 1 × 10⁵ and 2 × 10⁵ cells per 10 ml in 250-cm² tissue culture flasks. Cells were transferred into new flasks after 24 h and fed with 10 ml of fresh GM-CSF-supplemented medium after 4 days. DC were used after 6–8 days of culture. Macrophages were seeded in a density of 1 × 10⁵ cells/well in 96-well flat-bottom tissue culture plates and were fed with 100 μl GM-CSF-supplemented medium every 4 days. Macrophages were used after 12–16 days of culture and to remove non adherent cells were washed thoroughly before use.

ELISPOT-based Ag presentation assay

Ag presentation by in vivo infected cells was assessed with an ELISPOT-based Ag presentation assay as described previously (13, 14). This assay applies the basic principle of the ELISPOT assay for the detection of Ag presentation by target cells that acquired Ag in vivo. Spleens were removed 24 h after i.v. infection of mice with either 1 × 10⁶ CFU L. monocytogenes wild type (wt) or 1 × 10⁴ CFU L. monocytogenes Δlyt. Macrophages and DC were isolated from spleens as described above. To ensure sufficient cell numbers, DC and macrophages were isolated from pools of splenocytes from three identically treated mice. Ag presentation tests were performed in the presence of 10 μg/ml gentamicin and 20 μg/ml tetracycline. In round-bottom 96-well microtiter plates, 3 × 10⁶ peptide-specific CD8 T cells were added per well to the same number of Ag-pulsed cells. After 4 h predetermination, cells were resuspended, and 100 μl cell suspension were transferred to rat anti-mouse IFN-γ mAb-coated (RMMG-1; Biosource, Camarillo, CA) nylon membrane-backed 96-well microtiter plates (Nunc, Wiesbaden, Germany) and incubated overnight. ELISPOT plates were developed with biotin-labeled rat anti-mouse IFN-γ mAb (clone XM1G-2; BD Biosciences), HRP-streptavidin conjugate (Dianova, Hamburg, Germany), and aminomethylcarbazole dye solution.

Ex vivo enumeration of peptide-specific CD8 T cells

The frequency of peptide-specific CD8 T lymphocytes was determined in an IFN-γ-specific ELISPOT assay as described previously (8). Mice were infected i.v. with either 1 × 10⁶ CFU L. monocytogenes wt or 1 × 10⁴ CFU L. monocytogenes ΔactA. Unseparated splenocytes (6 × 10⁷/well) were stimulated for 6 h in round-bottom 96-well microtiter plates in the presence of 100 μg/ml peptide. Supernatants were harvested 24 h after stimulation and 100 μl/well were transferred to anti-IFN-γ-coated ELISPOT plates which were developed as described above. The frequency of Ag-specific cells was calculated as the number of spots per splenocytes seeded. The specificity and sensitivity of the ELISPOT assay were controlled with IFN-γ-secreting CD4 and CD8 T cell lines specific for LLO0-99 and LLO0-100, respectively. Recovery of seeded T cells was generally higher than 90%.

Cross-presentation assay

Per well of a 96-well microtiter plate, 50,000 macrophage-like P388D₁ (P388), cell lines were seeded in 200 μl 10% DMEM without antibiotics supplemented with 100 U/ml IFN-γ. After 24 h, adherent cells were washed once with PBS and were further cultivated in IFN-γ-free medium. Alternatively, macrophages were grown from bone marrow as described above. L. monocytogenes infection was performed for 1 h (bone marrow macrophages, 3 h) at a multiplicity of infection (moi) of 10:1 with centrifugal resuspension (10 min at 200 × g). After infection, APC were washed once with medium supplemented with 50 μg/ml gentamicin and once with medium supplemented with 10 μg/ml gentamicin and cultivated for further 6 or 18 h in 100 μl/well of medium supplemented with 10 μg/ml gentamicin. Loading of APC with HKL was performed as above but with an moi of 100:1. For the cross-presentation assay, MHC-mismatched DC or macrophages were grown from bone marrow or isolated from spleens of naive mice as described above and were added to P388 cells or bone marrow macrophages that were either infected with L. monocytogenes or loaded with HKL before. In some experiments, DC were fixed with 1% paraformaldehyde before use. The apoptosis inhibitor Z-Val-Ala-Asp-fluoromethyl ketone (ZVAD; Bachem, Heidelberg, Germany) and the inhibitor of the caspase-7, 72 (CDD, Sigma-Aldrich, St. Louis, MO) were added with DC in final concentrations of 500 μM and 10 μM, respectively. Controls with DC loaded with synthetic peptides were included to monitor potential direct toxic effects of these inhibitors on T cells. The lysosomal inhibitor chloroquine was used in a final concentration of 100 μM and was washed out before addition of T cells. Per well, 2 × 10⁵ DC or macrophages were added in 50 μl medium supplemented with 10 μg/ml gentamicin. After 1 h, finally 5 × 10⁵ T cells were added to the mixture of P388 and DC in 50 μl medium supplemented with 10 μg/ml gentamicin. Alternatively, T cells were added to P388 or DC only which were infected with L. monocytogenes or loaded with HKL as described above. After 18 h incubation at 37°C, the IFN-γ concentration in the supernatant was measured in an IFN-γ-specific ELISA that binds and detects IFN-γ with a pair of specific mAb. Results were corrected for dilution of the sample to yield the sample concentration in nanograms per milliliter.

Detection of necrotic and apoptotic cells

Macrophage-like P388 cells or DC grown from bone marrow cultures were either infected with viable L. monocytogenes wt (moi 10) or loaded with HKL (moi 100) for 1 h as described above. After 4 h the number of necrotic

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and apoptotic cells was assessed by staining with annexin V-FITC and propidium iodide (annexin V-FITC Apoptosis Detection kit; BD Biosciences). Necrotic cells are defined as propidium iodide positive, apoptotic cells as propidium iodide negative, annexin V positive.

Results

Synchronous expansion of LLO-specific CD4 and CD8 T cells

Most studies of the L. monocytogenes-specific T cell response focused on CD8 T cells. Because L. monocytogenes resides in the cytoplasm of host cells, it is generally accepted that antilisterial protection depends primarily on CD8 T cells and that the antilisterial T cell response is governed by CD8 T cells. Recently, the analysis of the LLO- and p60-specific T cell response in infected mice revealed that the H-2d-restricted T cell response in C57BL/6 mice is dominated by LLO91-201-specific CD4 T cells (8). This opposes the well-studied H-2d-restricted dominant CD8 T cell response in BALB/c mice (24). To test whether the strong H-2d-restricted CD8 T cell response can coexist with the H-2d-restricted CD4 T cell response against L. monocytogenes, B6CF1 (H-2b x H-2d) mice were studied. Interestingly, infected B6CF1 mice mounted a strong codominant CD4 and CD8 T cell response against L. monocytogenes LLO190-201 and p60367-378, respectively (Fig. 1A). The strongest p60-specific CD4 and CD8 T cell populations recognized p60367-378 and p60217-225, respectively.

To compare the expansion kinetics of L. monocytogenes-specific CD4 and CD8 T cell frequencies of T cells directed against the CD8 T cell epitope LLO91-201 and p60217-225 and the CD4 T cell epitope LLO190-201 and p60367-378 were monitored at various time points after infection (Fig. 1B). LLO- and p60-specific CD4 and CD8 T cell populations expanded synchronously between day 3 and day 10 after infection.

These results indicate that the T cell response against L. monocytogenes is not generally biased toward CD8 T cells as it could be predicted on account of the cytoplasmatic localization of L. monocytogenes.

Induction of LLO-specific CD4 T cells occurs actA independently

A previous study of the presentation of LLO-derived CD4 T cell epitopes revealed that the efficient MHC class II-restricted presentation of LLO itself depends on the actA-dependent intercellular spread of bacteria (9). To decide whether this mode of Ag presentation is also relevant for the induction of CD4 T cells in vivo, the T cell response against p60- and LLO-derived CD4 and CD8 T cell epitopes was compared after infection of mice with either L. monocytogenes wt or L. monocytogenes ΔactA, an actA in-frame deletion mutant of the wt strain (15). Because the virulence and the course of infection of L. monocytogenes wt and the ΔactA mutant differ strongly, it is not possible to prove that in both experimental settings the same total doses of LLO or p60 are produced in the infected host. However, CD8 epitopes derived from the p60 (10) and LLO (data not shown) of both Listeria strains are presented with similar strength after infection of P388 cells in vitro.

The frequencies of LLO91-201-specific CD4 T cells and LLO91-201-specific CD8 T cells were determined (Fig. 2A) and the ratio of CD4 to CD8 T cells was calculated (Fig. 2B). From the frequencies of p60367-378-specific CD4 T cells and p60217-225-specific CD8 T cells, the CD4:CD8 ratio of p60-specific T cells was calculated (Fig. 2, C and D). Mice infected with L. monocytogenes wt and L. monocytogenes ΔactA revealed similar CD4:CD8 ratios for the LLO- and p60-derived epitopes, respectively, indicating that the induction of CD4 T cells does not require actA expression in vivo.

Presentation of L. monocytogenes-derived CD4 T cell epitopes in vivo

Naïve T cells are most efficiently activated by Ag presented by DC (25). To assess Ag presentation by these professional APC, CD11c-positive cells, which are mostly DC, were isolated from spleens of infected mice and tested with a panel of peptide-specific T cell lines in a sensitive ELISPOT assay (13, 14). Fig. 3A shows the analysis of DC isolated from B6CF1 (H-2b x H-2d) mice infected with 1 x 10^5 CFU L. monocytogenes wt (Fig. 3A, top), 1 x 10^7 CFU L. monocytogenes ΔactA (Fig. 3A, middle), or 5 x 10^6 CFU L. monocytogenes Δhly (Fig. 3A, bottom), respectively. The background activity of APC was dependent on the number of APC seeded per well. The best signal-noise ratio was obtained with 1 x 10^4 to 3 x 10^3 DC per well. The specificity of the assay is illustrated by the analysis of DC from mice infected with the LLO-deficient L. monocytogenes Δhly strain, which were not recognized by two different LLO-specific CD4 T cell lines. DC isolated from spleens 24 h after i.v. infection of B6CF1 mice with 1 x 10^5 CFU L. monocytogenes wt efficiently presented the LLO-derived CD4 T cell epitopes LLO190-201, LLO253-264, LLO318-325, and also the CD8 T cell epitope LLO190-199 (Fig. 3B, top). The CD4 T cell epitope p60177-188 and the CD8 T cell epitope p60217-225 were also recognized by T cells of corresponding specificity. A similar peptide presentation pattern of p60 and LLO-derived CD4 and CD8 T cell epitopes was also observed 24 h after infection of mice with 1 x 10^5 CFU L. monocytogenes ΔactA (Fig. 3B, top), suggesting that MHC class II-restricted Ag presentation is not actA
dependent. In the absence of T cells, APC did not yield a significant spontaneous background activity, and no significant activation of murine CMV pp89–168-specific CD8 T cells occurred. Also, macrophages isolated from infected mice presented CD4 and CD8 T cell epitopes actA independently (Fig. 3B, bottom). Compared with DC, the Ag presentation pattern of macrophages was similar; however, activation of T cells was weaker.

Because differential effects of L. monocytogenes wt vs L. monocytogenes ΔactA infection on the expression of MHC class II (14) and costimulatory molecules could influence the results of the ex vivo Ag presentation assay, expression of costimulatory molecules on ex vivo isolated APC was monitored. After infection of mice with L. monocytogenes wt and L. monocytogenes ΔactA expression of MHC class II molecules, CD40, CD80, and CD86 on DC (Fig. 4, top) and macrophages (Fig. 4, bottom) was significantly increased compared with APC from naive mice, indicating that infection with L. monocytogenes wt and the ΔactA mutant have a similar effect on the expression of costimulatory molecules on professional APC in vivo.

These data indicate that L. monocytogenes-derived CD4 T cell epitopes are efficiently presented by professional APC in vivo.

Because the presentation of CD4 T cell epitopes compared with CD8 T cell epitopes was not influenced by actA, these results further suggest that the relevant presentation pathway is independent of the actA-mediated intercellular spread of L. monocytogenes and that it is also not inhibited by LLO.

**Cross-presentation of L. monocytogenes-derived CD4 T cell epitopes**

Because DC play a crucial role for the induction of a primary T cell response, the ability of DC to present proteins delivered by either heat-killed or viable L. monocytogenes was tested in an in vitro Ag presentation assay. DC were isolated from naive C57BL/6 mice and were either infected with L. monocytogenes or loaded with HKL. MHC class II-restricted Ag presentation was tested with a panel of L. monocytogenes-specific CD4 T cell lines (Fig. 5A, top). L. monocytogenes-infected DC were not recognized by LLO- or p60-specific CD4 T cells. After loading of DC with HKL, only a single CD4 T cell epitope, LLO190–201, of four epitopes tested was presented weakly. Thus, it seems unlikely that direct infection of DC results in strong presentation of L. monocytogenes-derived CD4 T cell epitopes in vivo.

**FIGURE 2.** Induction of a L. monocytogenes (LM)-specific CD4 T cell response is actA independent. A, The frequencies of LLO190–201-specific CD4 and LLO190–201-specific CD8 T cells were determined 10 days after primary infection of B6CF1 mice with either L. monocytogenes wt or L. monocytogenes ΔactA. B, From the frequencies of LLO190–201-specific CD4 T cells and LLO190–201-specific CD8 T cells, the CD4:CD8 ratio was calculated. C, The frequencies of p60367–378- and p60217–225-specific CD8 T cells were determined. D, The ratio of p60-specific CD4 and CD8 T cells was calculated. T cell frequencies were determined in an ELISPOT assay in the presence of 10^6 M concentrations of the indicated peptides. Data are from a representative experiment of three independent experiments that yielded similar results. Shown is the number of IFN-γ-positive cells per 1 × 10^6 splenocytes. Error bars indicate the SD of three individual mice tested.

**FIGURE 3.** Efficient presentation of L. monocytogenes-derived CD4 T cell epitopes by DC isolated from infected mice. Ag presentation by DC and macrophages isolated from CB6 F1 (H-2b) mice 24 h after infection was tested in the ELISPOT-based Ag presentation assay with L. monocytogenes-specific CD4 and CD8 T cell lines. APC were isolated from pools of splenocytes from three identically treated mice. A. DC were isolated from mice infected with L. monocytogenes wt (LM wt), L. monocytogenes ΔactA (LM ΔactA), or L. monocytogenes Δhly (LM Δhly), and graded numbers of DC were tested with T cells of the indicated specificity. B. Comparison of Ag presentation by DC (top) and macrophages (bottom) after infection of B6CF1 mice with L. monocytogenes wt or L. monocytogenes ΔactA. Ag presentation was tested with a set of L. monocytogenes-specific CD4 (LLO190–201, LLO253–264, LLO318–329, p60177–188) and CD8 (LLO91–99, p60217–225) T cell lines. CD8 T cells directed against p689168–176 were included as a specificity control. Shown is the total number of spots per 2 × 10^6 CD8 T cells and 1.1 × 10^4 APC per well. Results were confirmed in three independent experiments.
Cross-presentation is an alternative mode of Ag uptake and presentation that depends on the uptake of antigenic material from apoptotic or necrotic infected cells (26). To study cross-presentation of CD4 T cell epitopes, an Ag presentation assay was established that requires Ag uptake from primarily infected MHC-mismatched APC. First, macrophage-like P388 cells (H-2\textsuperscript{d}) were either infected with \textit{L. monocytogenes} or loaded with HKL and were tested with a panel of H-2\textsuperscript{d}- and H-2\textsuperscript{b}-restricted \textit{L. monocytogenes}-specific T cell lines (Fig. 5A, middle). Infected P388 cells were recognized by LLO\textsubscript{91-99}-specific CD8 T cells, indicating that efficient infection and MHC class I-restricted presentation occurred. However, corroborating previous reports (9, 10), infected cells were not recognized by p60\textsubscript{367-378}-specific CD4 T cells. If P388 cells were loaded with HKL, efficient presentation of p60\textsubscript{367-378} occurred, indicating that infection with viable bacteria inhibited MHC class II-restricted Ag presentation. To test the cross-presentation of \textit{L. monocytogenes}-derived CD4 T cell epitopes, freshly isolated DC from C57BL/6 mice (H-2\textsuperscript{b}) were added to P388 cells (H-2\textsuperscript{d}) that were infected with viable \textit{L. monocytogenes} or loaded with HKL 6 h before. Ag presentation was again monitored with H-2\textsuperscript{b}- and H-2\textsuperscript{d}-restricted T cell lines (Fig. 5A, bottom). In this setting, very strong presentation of the H-2\textsuperscript{b}-restricted CD4 T cell epitopes was observed.

**FIGURE 5.** Cross-presentation of \textit{L. monocytogenes}-derived CD4 T cell epitopes by DC. 

A. Top, DC were isolated from naive C57BL/6 mice (H-2\textsuperscript{b}) and were either infected with \textit{L. monocytogenes} or loaded with HKL. Ag presentation was tested with a panel of H-2\textsuperscript{b}-restricted \textit{L. monocytogenes}-specific CD4 T cell lines (LLO\textsubscript{190-201}, LLO\textsubscript{253-264}, LLO\textsubscript{318-329}, p60\textsubscript{177-188}). Middle, Macrophage (Mø)-like P388 cells (H-2\textsuperscript{d}) were either infected with \textit{L. monocytogenes} or loaded with HKL and tested with H-2\textsuperscript{d}-restricted \textit{L. monocytogenes}-specific CD4 T cell lines. Bottom, Cross-presentation of CD4 T cell epitopes was analyzed after addition of DC (H-2\textsuperscript{b}) to P388 (H-2\textsuperscript{d}) cells that were either infected with \textit{L. monocytogenes} or loaded with HKL 6 h before. Cross-presentation was tested with H-2\textsuperscript{b}-restricted \textit{L. monocytogenes}-specific CD4 T cell lines. 

B. Comparison of the ability of ex vivo isolated DC and macrophages to cross-present \textit{L. monocytogenes}-derived Ag. P388 (H-2\textsuperscript{d}) cells were infected with \textit{L. monocytogenes} and DC or macrophages isolated from naive C57BL/6 mice (H-2\textsuperscript{b}) were added 18 h later. Cross-presentation was tested with H-2\textsuperscript{b}-restricted \textit{L. monocytogenes}-specific CD4 T cell lines. Similar results were obtained in three independent experiments.
epitopes p60<sub>177–188</sub>, LLO<sub>318–329</sub>, LLO<sub>253–264</sub>, and LLO<sub>190–201</sub> occurred. Strong cross-presentation of the CD4 epitopes occurred also with HKL-loaded noninfected P388 cells as source of Ag. Also the cross-presentation by macrophages was tested. Either DC or macrophages isolated from noninfected C57BL/6 mice were added to P388 cells 24 h after infection with <i>L. monocytogenes</i>. The strength of cross-presentation was quite similar for macrophages and DC (Fig. 5B). Similar results were also obtained with HKL-loaded P388 cells as source of Ag (data not shown).

The use of MHC-mismatched macrophages and DC allows the simultaneous measurement of direct Ag presentation and cross presentation but represents an artificial situation. Therefore, cross-presentation was also tested in a syngeneic system (Fig. 6). MHC-matched macrophages and DC were grown from bone marrow of B6CF<sub>1</sub> mice. Compared with macrophages loaded with HKL, macrophages infected with viable <i>L. monocytogenes</i> were poorly recognized by LLO<sub>190–201</sub>-specific CD4 T cells. Infection with <i>L. monocytogenes</i> also inhibited presentation of HKL that were simultaneously added to APC. Addition of DC resulted in much stronger activation of LLO<sub>190–201</sub>-specific CD4 T cells.

In summary, these experiments show that cross-presentation of antigenic material acquired by DC or macrophages from infected P388 cells circumvented the LLO-mediated blocking of MHC class II-restricted Ag presentation.

**Requirements for cross-presentation of L. monocytogenes-derived CD4 T cell epitopes**

From a number of studies, it is known that DC take up and cross-present material from apoptotic and necrotic cells (reviewed in Ref. 26). The possible induction of apoptosis and necrosis of APC was evaluated after either infection with viable <i>Listeria</i> or loading with HKL (Fig. 7). Compared with P388 cells, DC were more resistant to <i>L. monocytogenes</i>-induced cell death. After loading of P388 or DC with HKL, the number of apoptotic cells was <2% and equal to the percentage of apoptotic cells in untreated controls. These results indicate that poor presentation of <i>L. monocytogenes</i>-derived Ag by DC cannot be due to rapid cell death and further raise the question why HKL-loaded macrophages were efficiently cross-presented despite unchanged viability of APC.

To analyze whether cross-presentation requires direct contact to DC and active rearrangement of the cytoskeleton of DC inhibition studies with CDD were performed. Both the fixation of DC with paraformaldehyde and the addition of CDD to DC completely abrogated cross-presentation of the immunodominant CD4 T cell epitope LLO<sub>190–201</sub> (Fig. 8A). The requirement of direct cell to cell contact was further corroborated by the observation that the supernatant from infected macrophages could not sensitize DC for recognition by LLO<sub>190–201</sub>-specific T cells. In accordance with the observation that neither viable <i>Listeria</i> nor HKL induced apoptosis in macrophages, also the supplementation of cultures with the apoptosis inhibitor ZVAD did not significantly reduce cross-presentation (Fig. 8A). As indicated by the efficient recognition of synthetic LLO<sub>190–201</sub>, the inhibitors did not exert a direct toxic effect on responder T cells.

To analyze the effect of macrophages on the costimulatory activity of DC (27), the presentation of synthetic LLO<sub>190–201</sub> by B6CF<sub>1</sub> DC was measured in the presence and absence of MHC-mismatched CBA bone marrow macrophages. Coculture of DC with CBA macrophages significantly improved the recognition of peptide-loaded DC by the LLO<sub>190–201</sub>-specific CD4 T cell line (Fig. 8B). If DC were cocultured with macrophages that were loaded with HKL prepared from a LLO-deficient <i>L. monocytogenes</i> Δfly strain, no further up-regulation of the costimulatory potential of DC occurred (Fig. 8B). Thus, <i>Listeria</i> components were not required to up-regulate the costimulatory activity of DC.

To test whether during cross-presentation processing of Ag by macrophages is required, lysosomal Ag presentation in macrophages was inhibited with chloroquine. Chloroquine treatment totally abrogated presentation of LLO<sub>190–201</sub> and p60<sub>367–378</sub> after loading of CB6 macrophages with HKL (Fig. 9). In the cross-presentation experiment, CBA macrophages were either loaded with HKL or infected with <i>L. monocytogenes</i>. Infection with <i>L. monocytogenes</i> was performed for 4 h with an bacteria-cell ratio of 10. During this period, >90% of cells were killed (data not shown). Chloroquine was present during infection of macrophages but was removed before addition of DC to exclude a direct effect of chloroquine on DC. As shown in Fig. 9, chloroquine treatment of macrophages did not influence cross-presentation of MHC class II-restricted T cell epitopes by the DC.
of T cell studies focused on the *L. monocytogenes*-specific CD8 T cell response, which is particularly important for the protection of immune mice (28). Only recently has it become clear that the T cell response against *L. monocytogenes* is not generally dominated by CD8 T cells but comprises a strong CD4 T cell response (8, 29). The current study resolves this paradox by demonstrating that the suppressive effect of LLO on the *L. monocytogenes*-specific CD4 T cell response is not maintained in DC that cross-present *Listeria*-derived Ags.

Cross-priming was discovered during the study of the CD8 T cell response against minor histocompatibility Ag (30). These early studies showed that a CD8 T cell response could be induced with allogeneic APC, indicating that the Ag from the allogeneic APC is taken up and presented by recipient-type professional APC. For the involved mode of Ag presentation, the term cross-presentation was introduced (26). DC efficiently acquire and cross-present Ag acquired from apoptotic cells (31, 32). Cross-presentation has been described for a number of Ag, e.g., OVA (33), viruses (32), tumors (34), and bacteria (35). Generally, these studies focused on MHC class I presentation given that transfer of Ag can readily occur if only MHC class II presentation is involved. However, DC also process phagocytosed cell fragments onto MHC class II molecules if bacteria are grown under conditions that prevent induction of apoptosis in target cells (23). In contrast to previous reports (37), we did not observe apoptosis in *L. monocytogenes*-infected DC. Compared with P388 cells, DC were also relatively resistant against *Listeria*-induced cell death but were only poorly

**Discussion**

The murine infection with *L. monocytogenes* is a widely applied model for the analysis of T cell responses. However, the majority

**FIGURE 8.** Requirements for cross-presentation of *L. monocytogenes*-derived Ag by DC. Cross-presentation of *L. monocytogenes*-derived Ag from MHC-mismatched CBA (H-2b) bone marrow macrophages (Mφ) to bone marrow-derived B6CF1 (H-2b x H-2d) DC was measured with a LLO190-201-specific CD4 T cell line by quantification of IFN-γ secreted into the culture supernatant. A. DC were added to noninfected CBA macrophages (no Ag), or to macrophages infected with *L. monocytogenes* wt (LM), or to macrophages loaded with heat-killed *Listeria* (HKL). Cross-presentation by DC was tested in the presence of the apoptosis inhibitor ZVAD (ZAVD) or the inhibitor of the cytoskeleton CCD. Alternatively, DC were fixed by treatment with 1% paraformaldehyde (PFA). Instead of direct cocultivation of DC with infected macrophages, it was also tested whether cross-presentation occurs if only the supernatant of 24-h macrophage cultures is added to DC (SN). To exclude a possible toxic effect of APC on responder T cells, synthetic LLO190-201 was also added directly to DC/macrophage cocultures (peptide). B. DC grown from B6CF1 bone marrow were incubated with synthetic LLO190-201 peptide in the absence of macrophages (DC only), in the presence of noninfected CBA macrophages (Mφ + DC), or in the presence of CBA macrophages loaded for 3 h with heat-killed *L. monocytogenes* Δly (Mφ + DC + HKLΔly). As a specificity control, CBA macrophages were directly loaded with peptide (Mφ only). Shown is the IFN-γ concentration in nanograms per milliliter and the SD of triplicate determinations. Similar results were obtained in two independent experiments.

**FIGURE 9.** Cross-presentation does not require Ag processing by the primarily infected cell. Direct presentation of *L. monocytogenes*-derived Ag by B6CF1 (H-2b x H-2d) bone marrow macrophages and cross-presentation from MHC-mismatched CBA (H-2b) bone marrow macrophages to bone marrow-derived B6CF1 DC was measured in the absence and presence of chloroquine (Cq). Primary APC were either loaded with HKL (HKL) or infected with viable *Listeria* (LM). Chloroquine was present during the first 4 h after infection and was washed out before addition of DC. Ag presentation was measured with LLO190-201 and p60367-378-specific CD4 T cell lines by quantification of IFN-γ secreted into the culture supernatant. Shown is the IFN-γ concentration in nanograms per milliliter and the SD of triplicate determinations. Similar results were obtained in two independent experiments.
recognized by CD4 T cells after *L. monocytogenes* infection. Because intracellular bacteria could not be detected by staining of DC isolated from *L. monocytogenes*-infected mice and freshly isolated DC revealed an excellent viability (M. Škoberne and G. Geginat, unpublished observation), it is most likely that the majority of isolated DC were not infected by *L. monocytogenes* but acquired Ag by cross-presentation.

Also Ags from HKL were efficiently cross-presented, despite the fact that HKL did not induce necrosis or apoptosis in macrophages. The costimulatory activity of DC was up-regulated by noninfected macrophages as well as by macrophages loaded with HKL, indicating that the presence of *Listeria*-derived products was not required for the activation of DC. This is similar to a study of an MHC class II-restricted I-Ea-derived peptide (27) in which the cross-presentation of the I-Ea-derived epitope was not dependent on the induction of apoptosis or necrosis in the Ag-bearing cells. This observation was explained by the relatively high number of dead cells (20–40%) in the cultures. Similarly, we found that ~15% of P388 cells were necrotic and the functional analysis showed that these spontaneously dying cells were sufficient to increase the costimulatory activity of DC. In contrast to MHC class I-restricted cross-presentation of virus and *Salmonella*-derived Ag (32, 35), MHC class II-restricted cross-presentation did not require pathogen-induced apoptosis. Probably, for cross-presentation of MHC class I-restricted epitopes, the amount of Ag is more critical (26), which would explain why in these studies cross-presentation required pathogen-induced necrosis or apoptosis.

DC loaded with HKL were weak stimulators of CD4 T cells compared with DC that acquired HKL-derived Ag by cross-presentation. A possible explanation for the weaker recognition of DC loaded directly with HKL is that in the absence of necrotic or apoptotic cells the costimulatory activity of DC is not up-regulated as discussed above. Generally, HKL-loaded DC were also weaker stimulators of CD4 T cells than HKL-loaded macrophages or macrophage-like P388 cells. This difference was pronounced if HKL and APC were incubated shortly (1 h; see Fig. 5A), but after more extended incubation of APC and HKL (3 h; see Fig. 6) almost similar Ag presentation levels of LLO190–201 were obtained with DC and macrophages, indicating that DC are principally able to take up and present HKL-derived Ag in the context of MHC class II molecules. Because HKL were centrifuged on monolayers of adherent macrophages, it is also likely that macrophages were loaded with HKL more efficiently than DC that grow in suspension. Because both DC from day 6–8 bone marrow cultures and DC isolated from spleens of naive mice phagocytosed HKL (M. Škoberne and G. Geginat, unpublished observation), which indicates that both types of DC contain also immature DC, it seems unlikely that the presentation of HKL was limited by the maturation stage of DC.

From a number of model studies, it is known that macrophages also have the ability to cross-present Ag (34, 38), but because macrophages are not strong inducers of a primary T cell response they are not implicated in cross-priming of T cells (26). Macrophages isolated from spleens of infected mice also presented *L. monocytogenes*-derived CD4 T cell epitopes. However, activation of CD4 T cells by macrophages was significantly weaker than T cell activation by DC. This can be due to a lower level of Ag presentation, weaker expression of costimulatory molecules, or both. Because macrophages loaded with HKL in vitro presented LLO-derived, MHC class II-restricted antigenic peptides, presentation of Ag from killed bacteria is a possible presentation pathway that avoids the adverse effect of LLO on CD4 T cells. In addition, the in vitro analysis revealed that macrophages were also able to cross-present *L. monocytogenes*-derived Ag in the context of MHC class II molecules.

CD8 T cells directed against different *L. monocytogenes*-derived epitopes expand and contract synchronously after infection of mice (24). Also, *L. monocytogenes*-specific CD4 T cells expanded synchronously with CD8 T cells, indicating that the postulated general infectious stimulus driving expansion of CD8 T cells (24, 39) also acts on CD4 T cells. During the contraction phase of the T cell response, p60- and LLO-specific CD4 T cells contracted synchronously but compared with p60- and LLO-specific CD8 T cells with an accelerated kinetics, suggesting that apoptosis of CD4 and CD8 T cells is regulated differentially.

The mechanism by which LLO inhibits T cell recognition of infected APC is only partially understood. The study of LLO-treated APC loaded with model Ag revealed that the primary mode of action of LLO is not the inhibition of Ag processing or presentation but the induction of T cell anergy (12). This process requires the presence of the Ag and LLO. Cells treated with LLO alone also induce unresponsiveness in LLO-specific CD4 T cell lines. A possible mechanism of the LLO-mediated CD4 T cell suppression is that it influences the endosomal Ag-processing pathway in a way that finally results in the generation of modified, antagonistic T cell epitopes (12). The strong activation of CD4 T cells by DC isolated from spleens of infected mice and also the results of the in vitro Ag presentation assay indicate that DC that took up and cross-presented antigenic material from other infected cells were not affected by LLO. Because LLO is rapidly inactivated spontaneously (40), it is likely that the effect of LLO is maintained only in the primarily infected APC.

Second to LLO, also actA, a virulence factor of *L. monocytogenes* that enables intracellular and intercellular motility of bacteria by actin polymerization (41), is known to influence MHC class II-restricted Ag presentation. Using an in vitro intercellular spread assay, Hilboldt et al. (9) have shown that directly infected macrophages selectively present MHC class I-restricted antigenic peptides whereas APC infected indirectly by intercellular spread present both MHC class I- and MHC class II-restricted epitopes. The Ag presentation pathway relevant in this experimental setting was probably different to cross-presentation in that it required the intercellular spread of viable bacteria. The analysis of mice infected with either *L. monocytogenes* wt or *L. monocytogenes* ΔactA revealed that actA expression did not influence the relative strength of MHC class I- and MHC class II-restricted presentation of LLO- or p60-derived epitopes by DC or macrophages in vivo, and it also did not change the CD4:CD8 ratio of T cells directed against p60 and LLO. Because it is impossible to prove that after infection with both *Listeria* strains the same total doses of LLO or p60 were produced in vivo, MHC class II-restricted Ag presentation of p60 and LLO and cognate CD4 T cells were measured in relation to the presentation of MHC class I-restricted epitopes and the protein-specific CD8 T cell response. If actA expression would selectively influence MHC class II-restricted Ag presentation in vivo it should change the strength of MHC class II-restricted Ag presentation and the CD4 T cell response in relation to the strength of MHC class I-restricted Ag presentation and the CD8 T cell response.

The observation that DC, which play an important role as APC for the induction of a primary T cell response, cross-present *L. monocytogenes*-derived Ag has potential implications for the development of vaccines based on recombinant bacteria. These T cell vaccines should provide Ag in a form that results in optimal cross-presentation. The optimization of carriers and delivery systems for efficient cross-presentation of Ag is a possible new design rule and in vitro test criterion for T cell vaccines.
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References


