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ORIGINAL PAPER

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Human dendritic cells process and present Listeria antigens for in vitro priming of autologous CD4+ T lymphocytes

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Abstract The role of human dendritic cells (DC) in the immune response toward intracellularly growing Listeria was analyzed under in vitro conditions using several morphological and functional methods. DC incubated with *Listeria innocua* and *L. monocytogenes*, respectively, readily phagocytosed the bacteria. Listeria did not impair viability and immunogenic potential of human DC. Listerial antigens were found to be processed within the lysosomal compartment of DC and colocalized with major histocompatibility complex (MHC) class II molecules, as shown by fluorescence and transmission electron microscopy. DC challenged with apathogenic *L. innocua* were highly effective in priming autologous naïve T cells (mainly CD4+) in vitro. The T cells strongly proliferated in the presence of DC incubated with *L. innocua*, which could be significantly inhibited by anti-MHC II mAb. *L. innocua*-primed T cells were also successfully stimulated by DC harboring the pathogenic *L. monocytogenes*, either the wild-type strain EGD or the p60 reduced mutant strain RIII. From our results, we conclude that human DC infected with nonpathogenic intracellular bacteria are able to efficiently prime naïve T cells, which are then suitable for recognition of antigens derived from related virulent bacterial species. This in vitro human model provides an interesting tool for basic research in infectious immunology and possibly for a new immunotherapy.

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Introduction

Listeria monocytogenes are gram-positive, facultative intracellular bacteria. They may cause gastrointestinal disease after ingestion via contaminated food. *L. monocytogenes* can enter cells via internalin A- and B-mediated phagocytosis. In order to avoid the bactericidal effect of phagolysosomal fusion, Listeria disrupt the phagosomal membrane using listeriolysin and phospholipases and escape into the cytosol where they can replicate. By actin-based motility, Listeria spread from cell to cell (Cabanes et al. 2002; Cossart and Bierne 2001; Portnoy et al. 2002).

In a rat intestinal loop system, dendritic cells (DC) were identified as first cellular targets of infection with *L. monocytogenes* and found to be critically involved in subsequent transportation of the pathogen to the lymph nodes (Pron et al. 2001). DC are the most potent antigen-presenting cells and are crucial for the initiation of a specific T cell response against infectious agents. They are derived from CD34+ bone marrow cells that migrate, through the blood circulation, to most organs and tissues where they become immature DC. The immature DC are able to phagocytose and process antigens, including bacteria. Mature DC present antigens on the major histocompatibility complex (MHC) class I and II molecules to naïve T cells, and they induce a specific cellular immune response (Banchereau and Steinman 1998; Kapsenberg 2003).

Human DC are known to phagocytose *L. monocytogenes* and to subsequently mature (Paschen et al. 2000; Kolb-Mäurer et al. 2000, 2001, 2003a). It has been found that opsonization of *L. monocytogenes* with human serum significantly enhances phagocytosis of the DC via

FcγRIII (CD16)-mediated receptor uptake. The predominant opsonins seem to be antibodies (Abs) directed against the p60 protein, which is constitutively expressed also in the apathogenic *L. innocua*. This apathogenic Listeria species is ubiquitously present in our environment, especially in nutrition. Anti-p60 protein Abs induced by environmental challenge with *L. innocua* are postulated to bind *L. monocytogenes* also and thus enhance phagocytosis by the DC. This indicates a link between humoral and cellular immunity that could provide an important barrier against the spread of virulent *L. monocytogenes* via the blood stream, which may contribute to the generally low morbidity rates of listeriosis (Kolb-Mäurer et al. 2001).

In mice, the potential role of *L. innocua* for enhancement of *L. monocytogenes* p60-specific CD4+ and CD8+ T-cell memory has been demonstrated (Harty and Pamer 1995; Geginat et al. 1999; Škoberne and Geginat 2002). The intracellular fate of *L. monocytogenes* has also been investigated in many studies carried out in animal models (Vasquez-Boland et al. 2001; Portnoy et al. 2002; Park et al. 2004).

On the contrary, only few data exist on the cellular immune reaction to Listeria in humans (Bläuer et al. 1995; Guo et al. 1995; Paschen et al. 2000; Kolb-Mäurer et al. 2000, 2001, 2003a, 2003b; Neumann et al. 2003). Little is known about antigen presentation of Listeria-challenged DC to T lymphocytes. Especially, links between the environmental challenge with the apathogenic *L. innocua* and induction of a cellular cross-reactive immune response toward *L. monocytogenes* has not been demonstrated in humans to date. It is unknown whether human T cells can be primed by DC infected with the avirulent *L. innocua* and whether these T cells are capable of mounting an efficient cross-reacting response toward pathogenic *L. monocytogenes*. This approach could be of interest for a better understanding of the cellular immune response in listeriosis.

Even though severe listeriosis is rare, in immunocompromised patients, it may cause sepsis, encephalitis, and meningitis. Infected pregnant women may suffer from chorioamnitis, which usually leads to abortion (Vasquez-Boland et al. 2001). Very rarely, *L. monocytogenes* causes endogenous endophthalmitis, which demands surgical intervention and, in prolonged cases, might even lead to visual loss (Berger et al. 2004). To date, the recovery rate of listeriosis by antibiotics is only approximately 70% (Hof 2004). One reason seems to be that antibiotics only poorly penetrate the cerebrospinal fluid and thus only high doses over a long period are curative. Furthermore, the intracellular localization of Listeria impairs the access to antibiotics. Immunomodulation to reconstitute immune defense capacity could presumably help to improve these conditions (Hof 2004).

This in vitro study was intended to characterize antigen presentation by human DC infected with both *L. innocua* and *L. monocytogenes*. First, we tested the uptake and influence of various Listeria species on DC function. Second, we investigated the antigen processing and presentation by the DC and intracellular fate of the Listeria. The third part dealt with the generation of specific T cells using autologous DC infected with *L. innocua* and testing their specificity on the pathogenic *L. monocytogenes*. We also analyzed potential antigens for cross-reactivity using a wild-type *L. monocytogenes* strain (EGD) and a p60-reduced rough mutant strain (RIII) and *L. innocua*, which are naturally lacking all PrfA-regulated virulence factors, including listeriolysin.

Materials and methods

Generation of DC

Human DC were generated in vitro from blood-derived precursors, as already described (Filgueira et al. 1996). Briefly, human peripheral blood mononuclear cells (PBMC) obtained from venous blood of healthy donors (Blood Bank SRK, Zürich, Switzerland) were isolated by Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) density centrifugation. The PBMC were cultured in RPMI 1640 supplemented with penicillin/streptomycin (All Life Technologies, Paisley, UK) and 10% heat-inactivated pooled human A serum (Blood Bank SRK) for 2 h. The adherent cells were further cultured in RPMI 1640 containing 5% heat-inactivated pooled human A serum, recombinant human granulocyte monocyte colony-stimulating factor (rhGM-CSF, 50 ng/ml, Novartis, Basel, Switzerland), and interleukin-4 (rhIL-4, 100 U/ml, R&D Systems, Abingdon, UK) for 6 days.

Infection of DC with Listeria

Listeria species and strains (see Table 1) were stored as 1-ml aliquots at −80°C. For experiments, a single aliquot was propagated in 50 ml of tryptic soy broth (Difco Laboratories, Detroit, MI, USA) at 37°C with aeration in a shaker incubator at 300 rpm for 16 h (Bläuer et al. 1995). DC were infected with Listeria opsonized for 30 min at 37°C in a mixture of 50% RPMI 1640 and 50% pooled heat-inactivated human A serum. For challenge, 10^8 bacteria were added to 5×10^5 DC, giving a multiplicity of infection (MOI) of 200 bacteria per DC (six-well tissue culture plates, Costar, NY, USA). After 30 min of co-incubation, the cell culture was washed with phosphate-buffered saline (PBS) and incubated overnight in culture medium supplemented with 100 μg/ml of gentamicin (Sigma, Buchs, Switzerland). The following day, DC were rinsed with PBS and resuspended in culture medium containing 50 μg/ml of gentamicin before use for the different experiments.

The viability and immunogenic potential of DC

The viability of DC after infection with the different Listeria species and strains was examined by a fluores-

Table 1 Listeria species and strains used

Strain	Genotype	Virulence factors/regulation	Reference
Listeria monocytogenes			
EGD	Wild-type	All virulence factors/PrfA P60/iap	Schaffner 1985
RIII	p60 Mutant	All virulence factors/PrfA p60 greatly reduced	Bubert et al. 1992
Listeria innocua			
LL-263	Wild-type	No virulence factors P60/iap	Bille and Rocourt 1996

cence release assay (Vollenweider et al. 1994). In brief, DC were infected, in 96-well flat bottom plates (Nunc, Roskilde, Denmark), with the Listeria, incubated overnight with gentamicin, as already described and the next day labelled with BCECF/AM (5 μg/ml, Nova Biochem, Lucerne, Switzerland) for 2 h. Unchallenged DC served as low control and triton-lysed DC as high control. The fluorochrome release from live DC was detected with Cytofluor 2300 (Millipore, Volketswil, Switzerland) and the viability calculated by the formula:

Percentage of living DC = (experimental value – triton-lysed DC)/(untreated DC – triton-lysed DC)×100.

In order to evaluate the influence of Listeria infection on DC surface marker expression, DC were tested by flow cytometry (Becton-Dickinson, San Josè, CA, USA). Fluorochrome-conjugated monoclonal antibodies (mAbs) directed against human CD80, CD83, and MHC II (HLA-DR, DP, and DQ, all PharMingen, Basel, Switzerland), CD14 (Diatec, Oslo, Norway), and CD16 (Becton-Dickinson) were used. A mouse anti-γ1 Ab (Becton-Dickinson) served as isotype control. For CD86 staining, we used an mAb purchased from Serotec Ltd. (Kidlington, Oxford, UK) followed by an FITC-labeled polyclonal goat anti-mouse Ab (Kirkegard & Perry Laboratory, Gaithersburg, MD, USA). All Abs were incubated at 4°C for 15 min (dilution 1:50).

Fluorescence microscopy

The adherent DC were fixed in six-well tissue culture plates with 3% paraformaldehyde and 2% sucrose in PBS, scraped with a cell scraper (Costar), and spun onto glass slides. Permeabilization was performed by placing in a microwave for 10 min at 900 W in 150 ml PBS. Thereafter, PBS containing 0.1% bovine serum albumin and 2% normal goat serum was used as blocking-buffer solution. Nuclear and bacterial DNA were stained with DAPI (4,6-diamidine-2-phenyl-indol-dihydrochlorid, 5 μg, Roche, Mannheim, Germany). Listeria were detected with a polyclonal anti-Listeria Ab (Fehr et al. 1997) kindly provided by Dr. Bille (WHO Collaborative Center for Foodborne Listeriosis, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland). A Texas red conjugated polyclonal goat anti-rabbit Ab (Jackson Immuno Research Laboratories, West Grove, PA, USA) was used as secondary Ab. For double immunofluorescence, the phagolysosomes of the DC were stained with a mouse mAb directed against the lysosomal-associated membrane protein LAMP-1 (CD107a, PharMingen), a late phagosome maturation marker (Chang et al. 2002; de Saint-Vis et al. 1998). In another double-immunofluorescence protocol, the MHC II molecules were visualized by an mAb directed against human MHC II (HLA-DR, DP, and DQ; PharMingen). For detection of intracellular MHC II colocalized with Listeria, we used a microwave, as already described. For simultaneous staining of membrane-bound MHC II with intracellular Listeria, we performed cell permeabilization by Triton X100 treatment (1% in PBS, 40 s, room temperature) because with this procedure, the membrane-bound MHC II molecules remained intact. Mouse mAb addition was followed by a polyclonal biotin-coupled sheep anti-mouse Ab (Amersham Pharmacia Biotech, Dübendorf, Switzerland) and FITC-labeled streptavidine (Bio-Science, Emmenbrücke, Switzerland). All Abs and reagents for staining were diluted to a previously tested optimal concentration in blocking-buffer solution. Exclusion of the primary Abs and noninfected DC served as negative controls. The specimens were analyzed as 0.2-μm-thin optical sections with a confocal laser-scanning microscope (CLSM, Leica, Heidelberg, Germany) using the Imaris software (Bitplane AG, Zürich, Switzerland).

Transmission electron microscopy

Listeria-harboring DC were prefixed in 0.1 M cacodylate buffer containing 2.5% glutaraldehyde and 0.8% paraformaldehyde, scraped, postfixed with an aqeuous solution of 1% OsO_4 and 1.5% $K_4Fe(CN)_6$, and embedded into epon. Transmission electron microscopy (TEM) analysis of ultrathin (approximately 50 nm) sections contrasted with lead citrate and uranyl acetate was performed with a CM 100 (Philips, Eindhoven, The Netherlands).

Generation and characterization of Listeria-specific T lymphocytes

For generating Listeria-specific T cells, we co-incubated in-vitro-generated DC from three healthy donors with *L. innocua*. Briefly, DC were infected with *L. innocua* at MOI of 200 for 30 min, rinsed, and cultured overnight with gentamicin to kill extracellular bacteria. The following day, freshly isolated nonadherent autologous

PBMC (5×10^6/ml) were added to the DC (10^5/ml in six-well plates). PBMC were cultured in RPMI 1640 containing 5% pooled human A serum and antibiotics under weekly restimulation. No additional cytokines were used for the first 3 weeks. After 3 weeks, 100 U/ml rhIL-2 (R&D Systems) were added. Cytofluorometry of the T lymphocytes was performed using FITC-labeled anti-human CD4, PE-coupled anti-human CD8 (both Serotec Ltd.), FITC-labeled anti-human CD3, PE-coupled anti-human CD16, and anti-human CD56 (all Becton-Dickinson) Abs on day 0 and after 2 weeks and 3 weeks in culture.

Specificity assays

Specificity of the T lymphocytes primed by use of autologous DC was tested after 3 weeks of stimulation in proliferation assays, as already described (Filgueira et al. 1996). Briefly, proliferative response of the T cells was measured by application of a 12-h pulse with 6−^{3}H-thymidine (0.5 mCi/well, specific activity 20 Ci/mmol, Amersham Pharmacia Biotech). Autologous DC either loaded with inactivated (gentamicin or heat) *L. monocytogenes* or with vital *L. innocua* were washed and cocultured for 3 days with the Listeria-specific T cells (in triplicates, 10^5 T cells and 10^4 DC per well, 96-well round bottom plates, Nunc). Thereafter, cells were harvested with the LKB harvesting system using glass fiber filter mats, and the incorporated 6−^{3}H-thymidine was detected using the beta-plate liquid scintillation counting system (all components LKB, Wallac Oy, Turku, Finland).

To analyze antigen presentation on MHC II molecules of the virulent species also, the escape of *L. monocytogenes* from the DC phagosomes was suppressed by treatment with gentamicin (300 μg/ml) prior to infection. Successful deactivation of the Listeria was assured in an overnight culture in 50 ml of tryptic soy broth where untreated *L. monocytogenes* could be successfully propagated. Cocultivation of the Listeria-specific T cells with untreated DC and of naïve PBMC with Listeria-infected DC served as negative controls. For positive control, T cells were cocultured with untreated DC in the presence of anti-CD3 mAb (clone CB3G) at maximal stimulatory concentration as nonspecific mitogenic stimulus (Filgueira et al. 1993). Furthermore, for blocking MHC class II antigen presentation, we used the mAbs directed against human MHC II (HLA-DR, DP, and DQ, PharMingen).

Statistical analysis

All results are expressed as mean ± standard deviation. Statistical analysis of the data was performed with a StatView program (Abacus Concepts Inc., Berkeley, CA, USA). This included an analysis of variance (Bonferroni/Dunn) with the significance level of $P < 0.01$.

Results

The viability and immunogenic potential of human DC after uptake of Listeria

In the first step, uptake of live Listeria species *L. innocua* and *L. monocytogenes* by human DC and intracellular localization were visualized by TEM. We found the already described zipper-like uptake for both Listeria species (compare Kolb-Mäurer et al. 2000) by the DC generated with our culture protocol (Fig. 1). Bacteria of both the tested strains (EGD and RIII) of *L. monocytogenes* were found free in the cytosol as well as within the vacuoles of DC. The p60-reduced strain RIII displayed the typical chain formation, indicating disturbed cell division, as already described (Minkowski et al. 2001). Furthermore, bacteria of all the Listeria species and strains investigated were found, by TEM, disrupted within phagolysosomes of the DC (data not shown). Thus, we were assured that DC generated with our culture protocol were capable of sufficient uptake of the Listeria at MOI of 200.

Secondly, we wanted to ascertain that DC challenged with Listeria were not functionally impaired in their viability and immunogenic potential. For that purpose, DC viability was tested 16 h after challenge with *L. innocua* and *L. monocytogenes* strain EGD by a fluorochrome release assay at MOI of 200 (Fig. 2a). Data were consistent with the results obtained by LDH release and MTT assays (data not shown). In all tests applied, Listeria-challenged DC revealed a high percentage of live cells.

In addition, the influence of Listeria uptake on DC maturation was assessed by flow cytometry (FACS) of relevant surface markers, including CD14, CD16, CD80, CD83, CD86, and MHC II. FACS analysis was performed on DC infected with vital *L. innocua* and *L. monocytogenes* strain EGD. The data revealed that both the DC population, with our in vitro culture protocol, were CD14−, CD16+, CD80+, and they strongly expressed CD83, CD86, and MHC II (Fig. 2b). Further tests were performed with DC infected with *L. monocytogenes* strain RIII and with DC coincubated with heat-inactivated bacteria (data not shown). All tests indicated that challenge with Listeria did not impair expression levels of the surface molecules tested in this study.

Intracellular processing and surface presentation of Listeria antigens

We further analyzed the intracellular processing of Listeria by confocal laser-scanning microscopy (Fig. 3). Using a polyclonal rabbit anti-Listeria Ab combined with a mouse mAb directed against a late phagosomal marker, the lysosomal-associated membrane protein LAMP-1, *L. innocua* were found with and without colocalization with LAMP-1, indicating presence of the Listeria inside and outside the phagolysosomes of

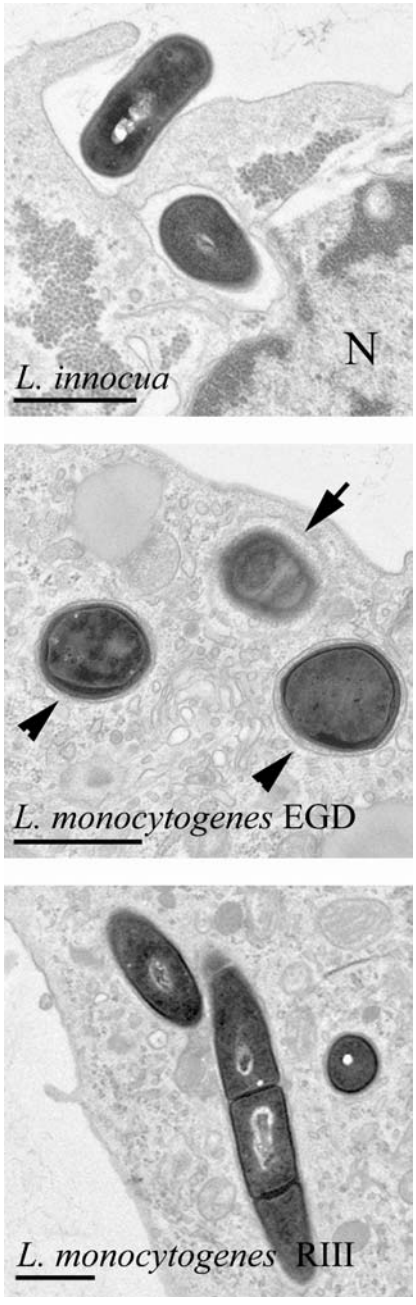

Fig. 1 Entry and intracellular localization of *Listeria innocua* and *L. monocytogenes* strains in dendritic cells (DC) visualized with transmission electron microscopy (TEM). *Top*: *L. innocua* enter DC by a zipper-like uptake. *N* Nucleus. *Middle*: *L. monocytogenes* strain EGD within vacuoles (*arrowheads*) and in the cytosol with beginning of actin polymerization (*arrow*). *Bottom* p60-reduced *L. monocytogenes* strain RIII revealing typical chain formation. *Bars* indicate 500 nm

the DC (Fig. 3, top row) Listeria without colocalized LAMP-1 might still be confined to the endosomes.

Moreover, double immunostaining of the DC by the anti-Listeria-Ab combined with a monoclonal Ab directed against human MHC class II (HLA-DR, DP, and DQ) displayed Listeria located within the phagolysosomes colocalized with MHC II molecules (Fig. 3, middle row). We further used the same combination of Abs with a modified permeabilization protocol (Triton) where the MHC II molecules at the cell surface remained intact. Thus, we were able to show that DC harboring *L. innocua* expressed MHC II molecules also at the cell surface surrounded by other PBMC, presumably T cells (Fig. 3, bottom row). Similar results were obtained with *L. monocytogenes* strain EGD (data not shown).

Phenotypic characterization of human T cells primed with autologous *L. innocua*-harboring DC

Distinct Listeria-specific T-cell populations were primed from freshly isolated human PBMC obtained from three healthy donors using autologous DC. For that purpose, DC were cocultured with *L. innocua* at MOI of 200 and used for weekly restimulation of PBMC to prime in vitro Listeria-specific T cells. Phenotypic characterization by flow cytometry of the PBMC before challenge indicated that the population consisted of about 35% natural killer (NK) cells (CD3−, CD16+, CD56+) and 65% T lymphocytes (CD3+). After 2 weeks in culture, the PBMC-derived population consisted of approximately 85% CD3+ T lymphocytes and 15% NK cells. After 3 weeks, NK cells had completely disappeared, and an approximately 100% CD3+ T lymphocyte population (first donor: CD4+: 90%, CD8+: 10%; second donor: CD4+: 80%, CD8+: 20%; third donor: CD4+: 30%, CD8+: 60%) was obtained. At that time point, specificity of the T lymphocytes from either donor was tested.

CD4+ T cells primed in vitro by *L. innocua*-challenged DC recognize Listeria-derived antigens presented by autologous DC

First, we tested whether the T lymphocytes primed in vitro in the presence of autologous *L. innocua*-challenged DC were capable of recognizing Listeria-infected DC. Furthermore, we were interested in the stimulatory capacity of the infected DC. For that purpose, the *L. innocua*-primed T cells were incubated in the presence of DC challenged with *L. innocua* (Fig. 4).

The *L. innocua*-primed T lymphocytes showed a significant proliferative response to DC infected with *L. innocua* (column 2; $P < 0.0001$), which was comparable to that with untreated DC in the presence of an unspecific mitogenic stimulus (anti-CD3 mAb; column 5). For negative control, we co-incubated the T cells with untreated DC (column 4), which did not significantly increase the basal proliferative activity of the T cells (column 6). The relatively high background activity of the T cells was probably due to our culture protocol where the T cells were primed by weekly restimulation with *L. innocua*-harboring DC from which residual stimulation might have arisen. Similar data were obtained from the other two donors (data not shown). As further controls, we also tested naïve autologous

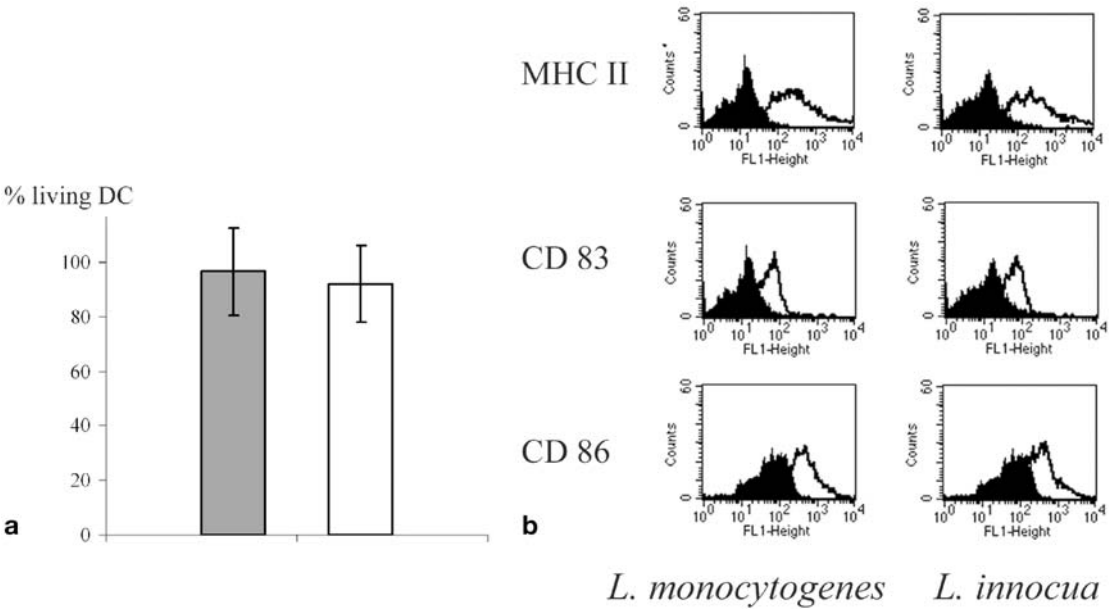

Fig. 2 Dendritic cell (DC) viability and immunogenic capacity after Listeria infection. DC were challenged with *Listeria monocytogenes* strain EGD and *L. innocua* at MOI 200 and analyzed after 16 h of further cultivation. **a** Quantification of fluorescence released by living cells in (%, *y axis*) was according to the formula described in Materials and methods. *Grey column*: DC infected with *L. monocytogenes* strain EGD; *white column*: DC infected with *L. innocua* (graph shows mean ± standard deviation = *error bars*). Representative from three independent experiments. **b** Cytofluorometric measurement of surface markers relevant for antigen presentation (MHC II, CD83, CD86). *Black curve*: isotype control; *white curve*: DC infected with *L. monocytogenes* or *L. innocua*

PBMC, which did not respond to the presence of Listeria-harboring DC (column 8) and tetanus toxin-specific T cells, which also did not respond to *L. innocua*-challenged DC (donor 3; data not shown). As a culture media control, T lymphocytes were cocultured with untreated DC in the presence of tryptic soy broth and gentamicin. No proliferative T-cell activity was induced (data not shown).

We further tested whether antigen presentation to the CD4+ T cells was mediated via MHC II molecules, as indicated by the morphologic studies. For that purpose, we used the mAb directed against human MHC II molecules (HLA-DR, DP, and DQ) to block antigen presentation by the DC. The proliferative response of the *L. innocua*-primed T cells in the presence of DC challenged with *L. innocua* could be significantly (column 3; $P < 0.0001$) inhibited with the MHC II mAb. On the contrary, treatment of T lymphocytes with the anti-MHC II mAb did not impair their proliferative activity (column 7). The results confirm MHC II-mediated specific antigen presentation already shown with fluorescence microscopy (Fig. 3). Similar results were also obtained using T cells obtained from donor 2 (data not shown).

Furthermore, we were interested to see if the *L. innocua*-primed T lymphocytes were also capable of recognizing the pathogenic *L. monocytogenes* (Fig. 5). We wanted to suppress the escape of bacteria from the phagosomes and thus support presentation of bacterial antigens by DC on MHC II molecules. For that purpose, we inactivated, prior to infection, the virulent *L. monocytogenes* using gentamicin or heat treatment. Efficient deactivation of the Listeria was controlled in an overnight culture, where no growth was detected.

The DC harboring gentamicin-inactivated *L. monocytogenes* strain EGD were able to induce a significant proliferative activity of the *L. innocua*-primed T cells (Fig. 5a, column 2; $P < 0.0001$) compared with untreated DC (column 4), indicating cross-reactive antigen recognition between the Listeria species mediated by DC. Similar results were obtained with heat-inactivated *L. monocytogenes* (data not shown). Further, we analyzed additional antigens possibly involved in cross-reactivity among *L. innocua* and *L. monocytogenes*. For that purpose, T lymphocytes were exposed to autologous DC co-incubated with the gentamicin-deactivated p60-reduced *L. monocytogenes* rough mutant strain RIII. p60 is a housekeeping protein constitutively expressed also by *L. innocua* and lacking all PrfA-regulated virulence factors such as internalin and listeriolysin, which *L. monocytogenes* possesses (compare Table 1). Also, DC harboring *L. monocytogenes* strain RIII were capable of a potent stimulation of the *L. innocua*-primed T cells (column 2; $P = 0.0002$) compared with untreated DC (column 4).

Also with the *L. monocytogenes* strains, the proliferative response could be significantly reduced (EGD: Fig. 5a, column 3; $P < 0.0001$; RIII: Fig. 5b, column 3; $P = 0.0003$) with the anti-MHC II mAb to the baseline proliferation level of the T cells.

Discussion

Listeria is a well-established model for studying the immune reaction against intracellular bacteria. It

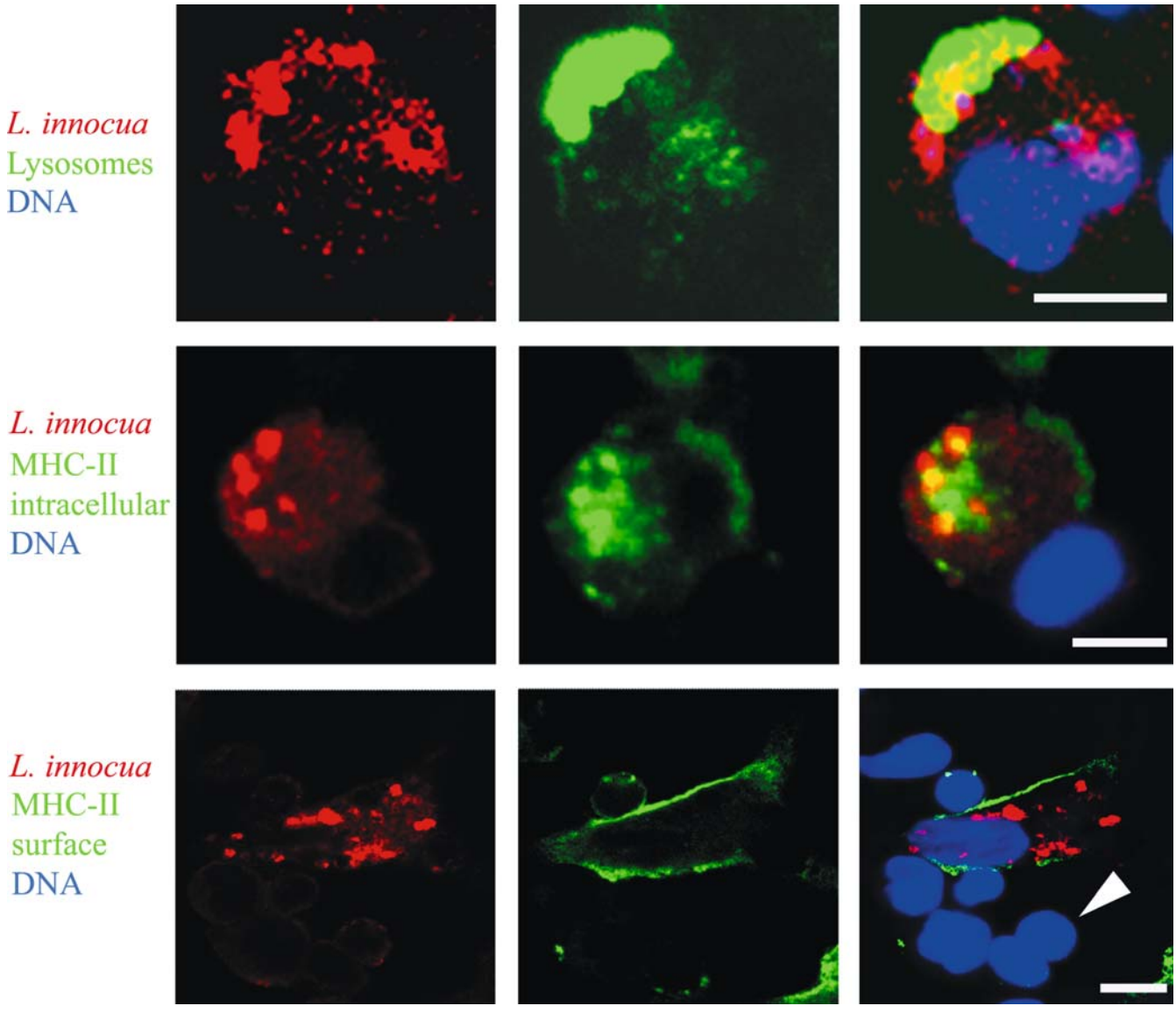

Fig. 3 Intracellular processing and presentation of *Listeria innocua* by dendritic cells (DC) examined with confocal laser-scanning microscopy. *Top row*: polyclonal antibody (Ab) directed against Listeria (*red*) detected with and without colocalization with monoclonal antibody (mAb) against LAMP-1 (*green*), indicating presence of bacteria within and outside the phagolysosomes. *Middle row*: polyclonal Ab directed against Listeria (*red*) colocalized with intracellular MHC II compartment stained with mAb (*green*). *Bottom row*: DC harboring *L. innocua* (*red*) expressing MHC II molecules stained with mAb (*green*) at the cell surface. DAPI-staining (*blue*) also shows nuclei of T cells (*arrowhead*), which are MHC-II-negative and do not contain *L. innocua*. *Bars* indicate 7 μm

consists of a large family of pathogenic and apathogenic species and is well characterized concerning genetic differences (Cabanes et al. 2002; Glaser et al. 2001). For these reasons, we chose Listeria as a model for the analysis: how DC act after challenge with intracellular pathogens. In our study, both the pathogenic and apathogenic Listeria species were phagocytosed by DC via the already described zipper-like uptake (see Kolb-Mäurer et al. 2000), transferred into endosomes (Fig. 1), processed in phagolysosomes (Fig. 3), and presented on MHC II molecules (Figs. 2, 3, 4, 5). Our findings confirm recent studies that showed DC to be highly tolerant toward infection with Listeria and unimpaired concerning antigen presentation (Paschen et al. 2000). In our experiments, at MOI of 200, DC revealed a strong immunogenic capacity and ability to efficiently prime in vitro specific T cells (Figs. 2, 3, 4, 5). This is in contrast to the results obtained in a mouse spleen DC cell line (CB1), which showed apoptosis of DC after *L. monocytogenes* infection (MOI 100, Guzman et al. 1995). Nevertheless, in a recent study in BALB/c mice, the cytokine gene expression of gastric tissue was functionally not impaired after *L. monocytogenes* infection (Park et al. 2004). For IL-12 and IL-18, produced by *L. monocytogenes*-infected human DC (see: Kolb-Mäurer et al. 2003a), gene expression was not reduced in the infected animals; on the contrary, it displayed a parallel increase with progressing

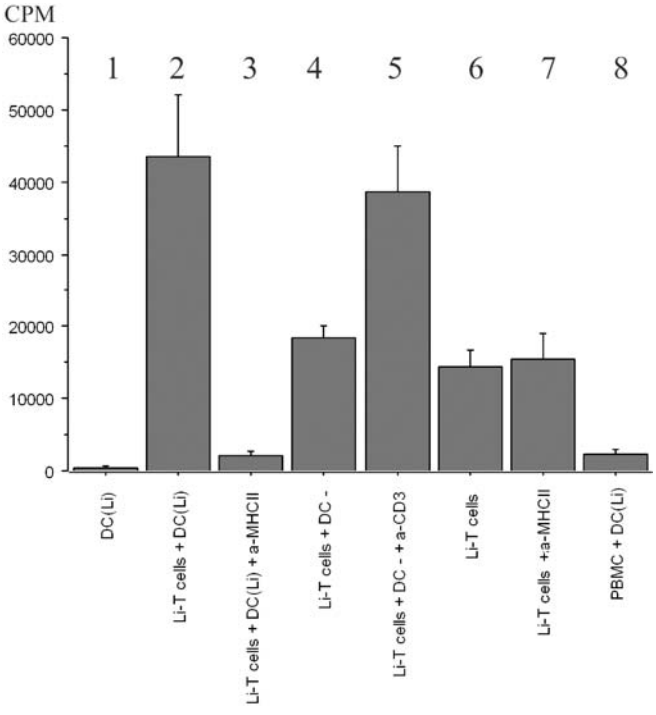

Fig. 4 Proliferative response of *Listeria innocua*-primed T cells (donor 1) after 3 weeks in culture (predominantly CD4+) exposed to autologous dendritic cells (DC) challenged with *L. innocua* (*Li*). *Column 1*: *L. innocua*-challenged DC. *Column 2*: *L. innocua*-primed T cells co-incubated with *L. innocua*-challenged DC. *Column 3*: Reaction from (*column 2*) blocked with anti-human MHC II monoclonal antibodies (mAb). *Column 4*: *L. innocua*-primed T cells co-incubated with untreated DC. *Column 5*: *L. innocua*-primed T cells cocultivated with untreated DC in the presence of anti-CD3 mAb as unspecific mitogenic stimulus. *Column 6*: *L. innocua*-primed T cells. *Column 7*: *L. innocua*-primed T cells treated with anti-MHC II mAb. *Column 8*: Naïve human peripheral blood mononuclear cells (PBMC) cocultured with *L. innocua*-challenged DC. ^{3}H-thymidine incorporation assay. *CPM* counts per minute. Means (± standard deviation, *error bars*) were calculated from triplicates

histopathological lesions (Park et al. 2004), which might indicate a similar active synthesis in the mouse DC also.

The pathogenic *L. monocytogenes* disposes over an apathogenic relative species, the *L. innocua* (Vasquez-Boland et al. 2001). Cross-reactivity between both the Listeria species mediated by opsonizing antibodies for enhanced phagocytosis has already been postulated (Kolb-Mäurer et al. 2001). However, a link between the humoral immune reaction towards *L. monocytogenes* induced by challenge with *L. innocua* and a cellular immune response has not been shown to date. The model of cross-reactivity of T lymphocytes primed using DC infected with an avirulent relative species, such as *L. innocua*, and recognition of common antigens on pathogenic relative species, such as *L. monocytogenes*, may contribute to a better understanding of environmentally provided protection against obligate or facultative intracellularly growing species (Kolb-Mäurer et al. 2001, 2003b).

In our in vitro study, we were able to prime specific T cells by the use of DC infected with avirulent *L. innocua*. Antigen presentation by the DC led to efficient T-cell priming of naïve PBMC and also to successful recognition of Listerial antigens by the T cells that could be blocked with an anti-MHC II Ab (Figs. 4, 5). The *L. innocua*-primed T cells were also capable of recognizing the pathogenic *L. monocytogenes* strain EGD when presented by DC, indicating successful cross-priming for both species (Fig. 5).

The predominant common antigen between the Listeria species is suggested to be the p60 protein, which is also constitutively found in *L. innocua*, in contrast to the PrfA-regulated virulence factors of *L. monocytogenes*, such as listeriolysin. The p60 protein, encoded by the *iap* gene, acts as an essential tool for cell viability and is required in a late step of cell division. It is the major extracellular protein of *L. monocytogenes* that contributes to its uptake into fibroblasts and macrophages (Kuhn and Goebel 1989; Bubert et al. 1992). In mice, listeriolysin and p60 protein from *L. monocytogenes* have been detected as main targets for CD4+ and CD8+ T cells (Brunt et al. 1990; Safley et al. 1991; Harty and Pamer 1995; Harty et al. 1996; Geginat et al. 1999; Škoberne and Geginat 2002). This is the reason we chose to additionally test the p60-reduced *L. monocytogenes* strain RIII (Bubert et al. 1992) where we found a strong cross-reactivity in the strain RIII, which again could be significantly blocked with a anti-MHC II mAb (Fig. 5), indicating specific bacterial antigen recognition (Kapsenberg 2003). However, it has to be considered that p60 synthesis is not completely abolished in this *L. monocytogenes* rough mutant strain but just greatly reduced (Bubert et al. 1992; Rowan et al. 2000). Thus, further studies are necessary to determine the possible antigens mediating cross-reactivity between *L. innocua* and *L. monocytogenes*. Common potential antigenic components of both Listeria species are under debate since there are only 68 genes encoding putative lipoproteins identified in *L. monocytogenes*, of which only six are absent in the *L. innocua* genome (Cabanes et al. 2002). The importance of bacterial lipoproteins for DC-mediated immune reaction has been recently shown in studies using *Borrelia burgdorferi* (Beermann et al. 2000).

From our results, we conclude that DC infected with nonpathogenic intracellular bacteria are able to survive infection, process Listerial antigens for presentation on MHC II molecules, and efficiently prime naïve PBMC to specific, mainly CD4+, T cells. These T cells may then even be suitable tools for the recognition of antigens of virulent relative bacteria species, which might provide an interesting approach for further research on the cellular immune response to bacteria. Recombinant *L. monocytogenes* is, in addition, an attractive tool as a vaccine vector, e.g., against human immunodeficiency virus (HIV). In a recent study, it could be demonstrated that oral immunization with recombinant *L. monocytogenes* controls virus load after vaginal challenge with feline immunodeficiency virus (FIV) (Stevens et al. 2004). Thus, further knowledge on Listeria's fate within DC

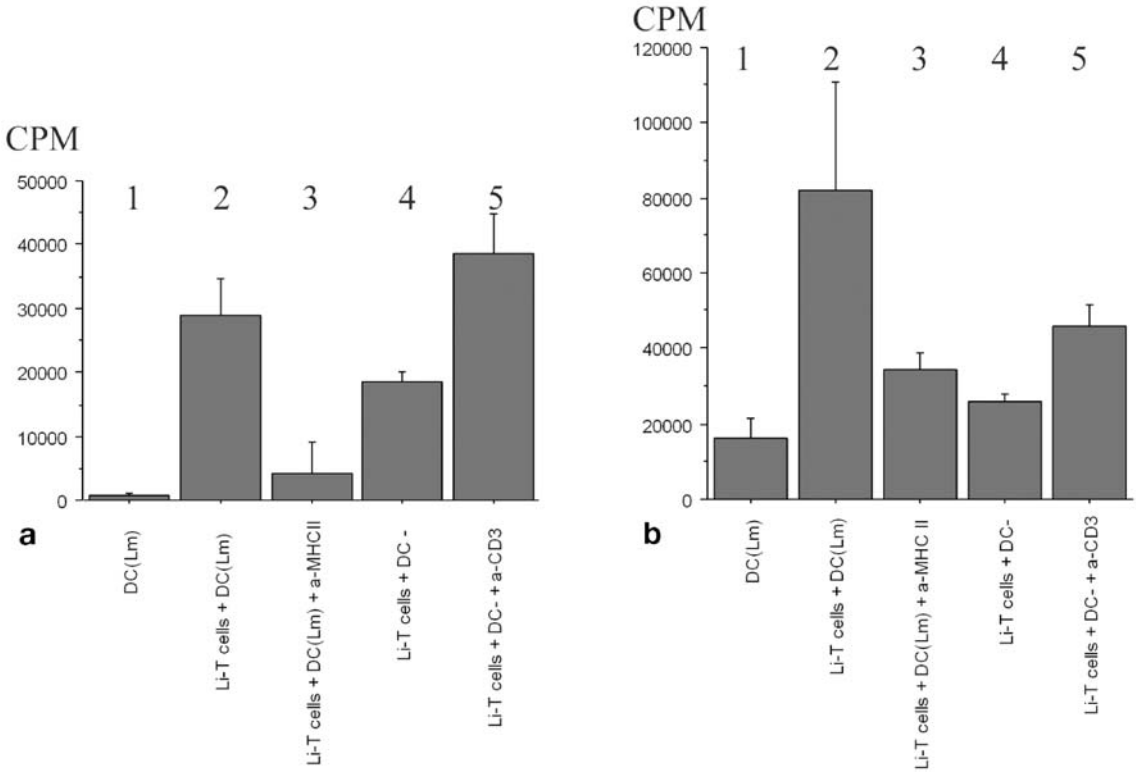

Fig. 5 Proliferative response of *Listeria innocua*-primed T cells after 3 weeks in culture (predominantly CD4+) exposed to autologous dendritic cells (DC) challenged with *L. monocytogenes* (*Lm*) inactivated by gentamicin prior to challenge. **a** Donor 1: DC co-incubated with Lm strain EGD. **b** Donor 2: DC co-incubated with Lm strain RIII. **a**, **b** (*Column 1*): Lm-challenged DC. *Column 2*: *L. innocua*-primed T cells co-incubated with Lm-challenged DC. *Column 3*: Reaction from (*column 2*) blocked with anti-human MHC II monoclonal antibody (mAb). *Column 4*: *L. innocua*-primed T cells co-incubated with untreated DC. *Column 5*: *L. innocua*-primed T cells cocultivated with untreated DC in the presence of anti-CD3 mAb as unspecific mitogenic stimulus. ^{3}H-thymidine incorporation assay. *CPM* counts per minute. Means (± standard deviation, *error bars*) were calculated from triplicates

might bring new insights into the processing of antigens in DC, in general.

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