CMLS, Cell. Mol. Life Sci. 62 (2005) 578–588
1420-682X/05/050578-11
DOI 10.1007/s00018-005-4518-4

CMLS Cellular and Molecular Life Sciences

Research Article

Legionella pneumophila down-regulates MHC class I expression of human monocytic host cells and thereby inhibits T cell activation

B. Neumeister[a, *], M. Faigle[a], D. Spitznagel[a], A. Mainka[a], A. Ograbek[a], H. Wieland[a], N. Mannowetz[a] and H.-G. Rammensee[b]

[1] Abteilung Transfusionsmedizin, AG Infektionsbiologie, Universitätsklinikum Tübingen, 72076 Tübingen (Germany), Fax: ++49 7071 295240, e-mail: Birgid.Neumeister@med.uni-tuebingen.de
[2] Interfakultäres Institut für Zellbiologie, Abt. Immunologie, Universität Tübingen, Auf der Morgenstelle 15, 72076 Tübingen (Germany)

Received 22 November 2004; received after revision 27 December 2004; accepted 5 January 2005

Abstract. *Legionella (L.) pneumophila,* the causative agent of Legionnaires' disease, is an intracellular pathogen of alveolar macrophages that resides in a compartment displaying features of endoplasmatic reticulum (ER). In this study, we show that intracellular multiplication of *L. pneumophila* results in a remarkable decrease in MHC class I expression by the infected monocytes. During intracellular multiplication, *L. pneumophila* absorbs ER-resident chaperons such as calnexin and BiP, molecules that are required for the correct formation of the MHC class I complex. Due to reduced MHC class I expression, stimulation of allogeneic blood mononuclear cells was severely inhibited by infected host cells but cytotoxicity of autologous natural killer cells against *Legionella*-infected monocytes was not enhanced. Thus, reduced expression of MHC class I in infected monocytes may resemble a new immune escape mechanism induced by *L. pneumophila*.

Key words: *Legionella*; MHC class I antigens; macrophages; natural killer cells; antigen presentation.

Legionella (L.) pneumophila, the causative agent of Legionnaires' disease, is an intracellular pathogen of alveolar macrophages. Pneumonia primarily occurs in immunocompromised hosts suffering from malignancies associated with monocyte dysfunction as well as in patients who receive immunosuppressive drugs. The mechanism by which *L. pneumophila* infection of the lung is controlled is not yet clear. *L. pneumophila* is relatively resistant to the innate and humoral immune response. The protective effect of antibody production and polymorphonuclear cells is limited [1–3] and activation of complement does not result in inactivation or killing of the bacteria [4]. Instead, cell-mediated immunity seems to be important for infection control. Initial investigations demonstrated that lymphocytes from convalescents undergo proliferation and release cytokine activity into the cell culture supernatant after in vitro contact with formalin-inactivated legionellae [5–10]. Macrophages that were activated by these cytokines were able to restrict the intracellular multiplication of legionellae after phagocytosis [11, 12]. Later on, this cytokine activity was identified to be interferon-gamma (IFN-γ) [13–18]. IFN-γ-activated macrophages restrict *L. pneumophila* growth by down-regulation of transferrin receptor and subsequent reduction of intracellular concentration of iron [17, 19–22] but are not able to kill all intracellular legionellae. Among the IFN-γ-producing cells, natural killer (NK) cells play a particular role. Resolution of replicative *L.*

* Corresponding author.

pneumophila infection via destruction of infected mononuclear cells has been mediated by interleukin (IL)-2-stimulated nonadherent peripheral blood leukocytes with features resembling these of of NK cells [23–25]. The trigger for this cytolytic NK cell activity remained unknown.

In a previous study, we demonstrated by immunocytochemistry the adsorption of resident endoplasmic reticulum (ER) proteins such as calnexin and BiP to *L. pneumophila* residing within human monocytes [26]. In this study, we provide evidence by electron microscopy and Western blot that adsorption of calnexin and BiP to *L. pneumophila* occurs during intracellular multiplication within human monocytes. Furthermore, we could show a remarkable decrease in MHC class I expression by the infected host cells leading to a suppressed stimulation of allogeneic blood mononuclear cells but not to an enhanced cytotoxicity of autologous NK cells against *Legionella*-infected monocytes. In addition to lipopolysaccharide possessing a dramatically reduced endotoxic capacity [27], the reduced expression of MHC class I in infected monocytes seems to be another immune escape mechanism of *L. pneumophila*.

Methods

Bacterial strains

L. pneumophila serogroup 1 substrain Pontiac (fresh human isolate from a patient with severe *Legionella* pneumonia) was kindly provided by Prof. Ruckdeschel (Munich, Germany). *L. pneumophila* serogroup 1 Philadelphia ATCC 33152 (isolated from human lung), *L. micdadei* ATCC 33218 (isolated from human blood via yolk sac), and *L. steigerwaltii* ATCC 35302 (isolated from tap water) were obtained from the American Type Culture Collection. Bacteria were grown on BCYEα agar (Biotest, Heidelberg, Germany) at 37 °C in 5% CO_2 for 3 days.

MonoMac 6 cells

MonoMac 6 cells (MM6) were kindly donated by Prof. H. W. L. Ziegler-Heitbrock (University of Leicester, UK) and cultured as previously described [28].

Preparation of human blood cells

Peripheral blood mononuclear cells (PBMCs) were prepared from leukocyte buffy coats, obtained from healthy blood donors after informed consent, by density centrifugation using Lymphocyte Separation Medium (PAA, Coelbe, Germany) according to the instructions of the manufacturer. For isolation of monocytes and NK cells, PBMCs of one donor were divided into two parts. Monocytes were negatively isolated from the first part using the Monocyte Isolation Kit (Miltenyi Biotec, Bergisch-Gladbach, Germany). Autologous NK cells were negatively selected from the second part of PBMCs using the NK Cell Isolation Kit (Miltenyi) according to the instructions of the manufacturer. Cell purity was assessed by flow cytometry analysis (FACScan; Becton Dickinson, Heidelberg, Germany). Based on the binding of anti-CD14-flourescein isothiocyanate (FITC) to monocytes (Serotec, Düsseldorf, Germany) and a two-color FACS analysis using anti-CD3-FITC and anti-CD56-phycoerythrin (PE) (Serotec) for NK cells, each cell population had a purity of 95 %.

Infection of human monocytes with *Legionella* species and intracellular growth

Infection of MM6 and PBMCs and the subsequent analysis of intracellular multiplication of *Legionella* species were performed as previously described [28]. Briefly, 2×10^7 monocytes were infected with 2×10^8 legionellae in a volume of 1.5 ml in a well of a six-well tissue culture plate (Greiner, Frickenhausen, Germany) to provide a bacterium to cell ratio of 10:1. After 2 h of coincubation, nonphagocytized bacteria were killed by the addition of 75 µg gentamicin per milliliter for 1 h at 37 °C in 5% CO_2. After three washes, the cells were distributed in 1-ml aliquots into the wells of a 24-well tissue culture plate (Costar), yielding a concentration of 2×10^6 infected monocytes per well. The cells were then incubated for 72 h at 37 °C in 5% CO_2 and the number of viable legionellae after multiplication in monocytes was determined every 24 h by hypotonic disruption of the cells and culture of the fluid on BCYEα agar. Six independent experiments were performed. Growth rates were determined statistically using the automatic spiral platers' evaluation pack (Meintrup DWS, Lähden-Holte, Germany).

FACS analysis of MHC expression of human monocytic cells

To examine the MHC expression of MM6 and monocytes prepared from PBMCs, FITC-labeled antibodies to human HLA class I and class II antigens (Serotec, Duesseldorf, Germany) were used and 10^6 cells per sample were analyzed with FACScan. Negative fluorescence was determined using cells stained with FITC-labeled antibodies of the relevant immunoglobulin isotype but of irrelevant specificities (Serotec). Results were expressed as percent positive cells as well as relative mean fluorescence intensity ± SD from at least three independent infections.

Allogeneic stimulation of PBMCs

PBMCs obtained from two unrelated blood donors were washed twice in RPMI 1640 (Invitrogen, Eggenstein, Germany). PBMCs from donor 1 (PBMC 1) were infected with *L. pneumophila* using a bacteria to cell ratio of 10:1. After incubation for 24 and 48 h, infected cells

were irradiated with 30 Gy (OB 29-4; STS, Braunschweig, Germany) and placed in 24-well plates (Costar; Corning Life Sciences, Schiphol-Rijk, The Netherlands) giving a concentration of 4×10^6 cells/well. Nonirradiated PBMCs (4×10^6) from donor 2 (PBMC 2) were added to each well. After incubation for 24, 48, and 72 h, cell culture supernatants were harvested and IL-2 was measured by enzyme immunoassay (R&D Systems, Wiesbaden, Germany). Uninfected, irradiated allogeneic PBMC 1 as well as irradiated, infected and uninfected autologous PBMC 2 served as controls. All experiments were performed at least in triplicate.

Determination of IFN-γ secretion in IL-2-activated NK cells

IL-2-activated NK cells were obtained after incubation of 2.5×10^6 NK cells/ml with 250 U/ml IL-2 for 48 h in RPMI plus 10% FCS. Autologous monocytes were infected with *L. pneumophila* or *L. steigerwaltii* using a multiplicity of infection (MOI) of 10:1 or 1:1 and were incubated for 24 and 48 h, respectively. Thereafter, monocytes were irradiated with 30 Gy and 2.4×10^5 infected monocytes were coincubated with 2.4×10^5 NK cells in RPMI plus 10% FCS. Noninfected monocytes served as a negative control. For control of maximal stimulation of the NK cells, 1.6×10^5 K562 cells or 25 ng/ml phorbol myristate acetate plus 2 µg/ml ionomycin (Sigma, Munich, Germany) were used. After 6 h coincubation at 37 °C in 5% CO_2, NK cells were measured for intracellular IFN-γ using a saponin/monensin method (Cytofix/Cytoperm Plus Kit; Becton Dickinson/PharMingen, Heidelberg, Germany) according to the instructions of the manufacturer. Cells were identified by staining with PerCP-labeled anti-CD3 (Becton Dickinson) and PE-labeled anti-CD56 (Serotec). Intracellular IFN-γ was detected by staining with FITC-labeled anti- IFN-γ (Becton Dickinson). Nonreactive monoclonal antibodies of the appropriate IgG isotype were used to stain the isotype controls. Stained cells were analyzed with FACScan. Results were expressed as percent IFN-γ-positive cells ± SD after subtraction of eventual positive signals from the isotype control immunoglobulin preparations. Three independent infections were performed. Samples were measured in duplicate.

Assessment of NK cell-mediated cytotoxicity

NK cell cytotoxicity was determined by performing a ^{51}chromium release assay. IL-2-activated NK cells (effector cells) were obtained after incubation of 2.5×10^6 NK cells/ml with 250 U/ml IL-2 for 48 h in RPMI plus 10% FCS. Autologous monocytes (target cells) infected with *L. pneumophila* using a MOI of 1:1 and 10:1, were incubated for 24 h. K562 cells, a NK-sensitive erythroblastoid leukemia cell line, served as a positive control, uninfected autologous monocytes served as a negative control. Before adding effector cells, infected and uninfected monocytes were labeled with $Na_2{}^{51}CrO_4$ (1.665×10^6 Bq) (Pharmacia, Freiburg, Germany) for 18 h. K562 cells were labeled for 1 h. The cells were washed and resuspended in RPMI containing 10% FCS. Target cells were placed in 96-well plates (Costar) giving a concentration of 5×10^3 cells/100 µl. Effector cells were added to provide an effector to target cell ratio of 50:1, 10:1 and 2:1. After incubation for 4 h at 37°C in 5% CO_2, the plates were centrifuged at 400 × g for 5 min and aliquots of the supernatant were analyzed in a gamma counter. Specific lysis was calculated as $(a - b/c - b) \times 100\%$, where a is experimental release, b is spontaneous release and c is maximum release. All experiments were done in triplicate.

Preparation of immunobeads

Dynabeads Pan Mouse M450 (Dynal, Hamburg, Germany) coated with the primary antibodies were coupled to the secondary IgG directed against *L. pneumophila* serogroup 1 lipopolysaccharide (mouse, monoclonal, clone LP46; Biotrend, Cologne, Germany) for 40 min at 4 °C. Beads were washed in PBS + 0.5% BSA.

Isolation of intracellular legionellae

To separate intracellular legionellae from MM6, infected monocytes were centrifuged at 400 × g for 5 min and washed twice with RPMI 1640. Lysis of MM6 and release of intracellular legionellae was undertaken by addition of 1 ml distilled sterile water to the cell pellet and homogenization of the monocytes by syringe and needle (0.2 mm). The crude cell fragments were removed by differential centrifugation at 400 × g for 5 min. For binding of the released intracellular bacteria, the antibody-coupled Dynabeads were mixed with the supernatant (about two beads per bacterium, calculated from intracellular bacterial growth rates) and incubated for 20 min at 4 °C with gentle shaking. Bacteria coupled to the beads were collected using a magnetic particle separator (Dynal) and washed three times with PBS/0.1% BSA (4 °C). Uninfected MM6 were treated in an identical manner and served as negative control.

SDS-PAGE and Western blot

Bacteria were lysed by addition of lysis buffer [29] and proteins were solubilized for 30 min on ice. Insoluble cell debris was sedimented by centrifugation (4 °C, 20 min, 22,000 g), the supernatants were concentrated at 4 °C using Microcon YM-10 filter devices (Millipore, Schwalbach, Germany) and 1/1000 (v/v) of protease inhibitor cocktail (Sigma) was added. Protein concentrations in the samples were determined using Roti-Nanoquant (Roth, Karlsruhe, Germany). Proteins (55 µg per lane) were subject to standard electrophoresis under reducing conditions on a 8% polyacrylamide gel. Ten micrograms of colored MagicMark protein standard (In-

vitrogen) served as molecular weight marker. For Western blotting, proteins were transferred to a nitrocellulose membrane (Sigma, Munich, Germany) by means of wet blotting at 300 V for 1.5 h (Hoefer Mini VE blot module, Pharmacia, Freiburg, Germany). The membranes were incubated at 4°C overnight in blocking solution (1% herring sperm in PBS) followed by incubation with the primary antibody [anti-calnexin, rabbit, polyclonal, Stressgen, Hamburg, Germany, 1:1000, in blocking solution; anti-BiP, goat, polyclonal, Santa Cruz, Santa Cruz, Calif. 1:1000, in blocking solution; anti-tapasin, goat, polyclonal, Santa Cruz, 1:1000, in blocking solution; anti-BiP (GrP78), goat, polyclonal, Santa Cruz, 1:1000, in blocking solution] for 1 h at room temperature. Membranes were washed with PBS (three times each, 5 min duration). Horseradish peroxidase (HRP)-labeled anti-rabbit IgG (Stressgen) or anti-goat IgG (Stressgen), 1:1000, was added and incubated for 1 h at room temperature. Membranes were washed again with PBS (three times each, 5 min duration). HRP was detected by addition of SuperSignal West Pico Chemiluminescent Substrate according to the manufacturer's instructions (Perbio Science, Bonn, Germany). For image acquisition and quantification, a digital imaging system (Diana III; Raytest, Straubenhardt, Germany) was used. Lysed uninfected MM6 cells served as positive control. Immunobeads incubated with lysed noninfected MM6 were used as negative controls.

Immunogold labeling of *L. pneumophila* and electron microscopy

Infected monocytes were lysed as described above. The crude cell fragments were removed by differential centrifugation at 400×g for 5 min and the resulting supernatants were centrifuged at 25,000 g for 30 min to enrich released bacteria. To localize *L. pneumophila* and to visualize adsorption of calnexin, the bacterial sediment was incubated with a mouse monoclonal antibody to *L. pneumophila* serogroup 1 LPS (anti-Lp46; Biotrend, Cologne, Germany) and with a rabbit polyclonal antibody to calnexin (Stressgen, Victoria, Canada), each diluted 1:5 in PBS/FCS (1%) for 30 min at 37°C with gentle shaking. After washing, bacteria were incubated with the secondary antibodies (goat anti-mouse, 10 nm gold conjugate, British Bio Cell, Cardiff, UK; and goat anti-rabbit, 5 nm gold conjugate, British Bio Cell) in the same manner as described above. Bacteria were fixed with 2.5% glutaraldehyde (Paesel+Lorei, Hanau, Germany) in 0.1 M cacodylate buffer for 2 h and washed with fresh buffer. After treatment with 1% OsO_4 (Paesel+Lorei) and saturated tannic acid solution (EMS, Fort Washington, Pa.), cells were harvested by centrifugation (400×g) and embedded in 2% agarose. Small cubes of agarose containing fixed cells were treated with 2% uranyl acetate (EMS) and washed with cacodylate buffer. Dehydration of the specimens was carried out with a graded ethanol series and propylene oxide. Finally, the probes were embedded in Araldite (Serva, Heidelberg, Germany) and polymerized. Semi- and ultrathin sections were cut on a Reichert ultramicrotome (Vienna, Austria) and mounted on pioloform coated copper grids (Storck Veco B. V., Eerbeek, The Netherlands). After contrast enhancement with lead citrate, the grids were examined using a Zeiss electron microscope (Zeiss, Jena, Germany).

Statistics

Groups were compared by one-way analysis-of-variance post-hoc tests performed by the Tukey-Kramer test.

Results

Legionella species differ in their intracellular multiplication rate

MM6 were infected with *Legionella* species and the intracellular growth rate was determined 72 h. The two strains of *L. pneumophila* were able to multiply within MM6, the fresh clinical isolate (Pontiac) showing a slightly higher intracellular multiplication rate than the agar-adapted strain (Philadelphia ATCC). Bacteria began to replicate immediately after in vitro infection and the number of intracellular bacteria increased by around 2.5 logarithms. In contrast, *L. micdadei* and *L. steigerwaltii* were not able to grow within MM6 (fig. 1). Monocytes obtained from peripheral blood mononuclear cells showed identical growth rates (data not shown).

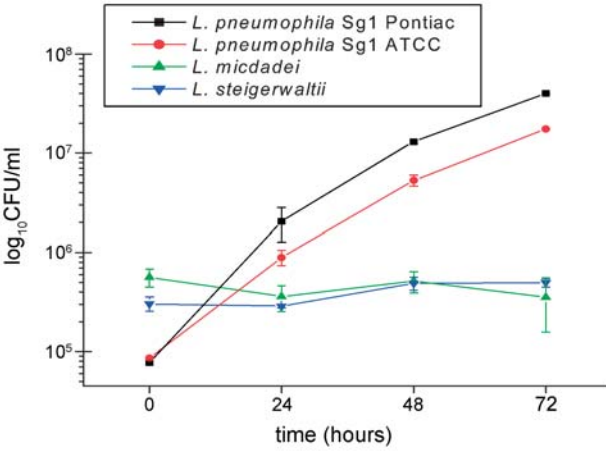

Figure 1. Intracellular multiplication of *Legionella* species within MM6. MM6 were infected using a bacterium to cell ratio of 10:1. After phagocytosis and killing of extracellular bacteria by gentamicin, cells were incubated for 72 h and the number of viable legionellae after multiplication in MM6 was determined by hypotonic disruption of the host cells and culture of the fluid on BCYEα agar. Growth curves show the mean intracellular number of bacteria ± SD of six independent experiments.

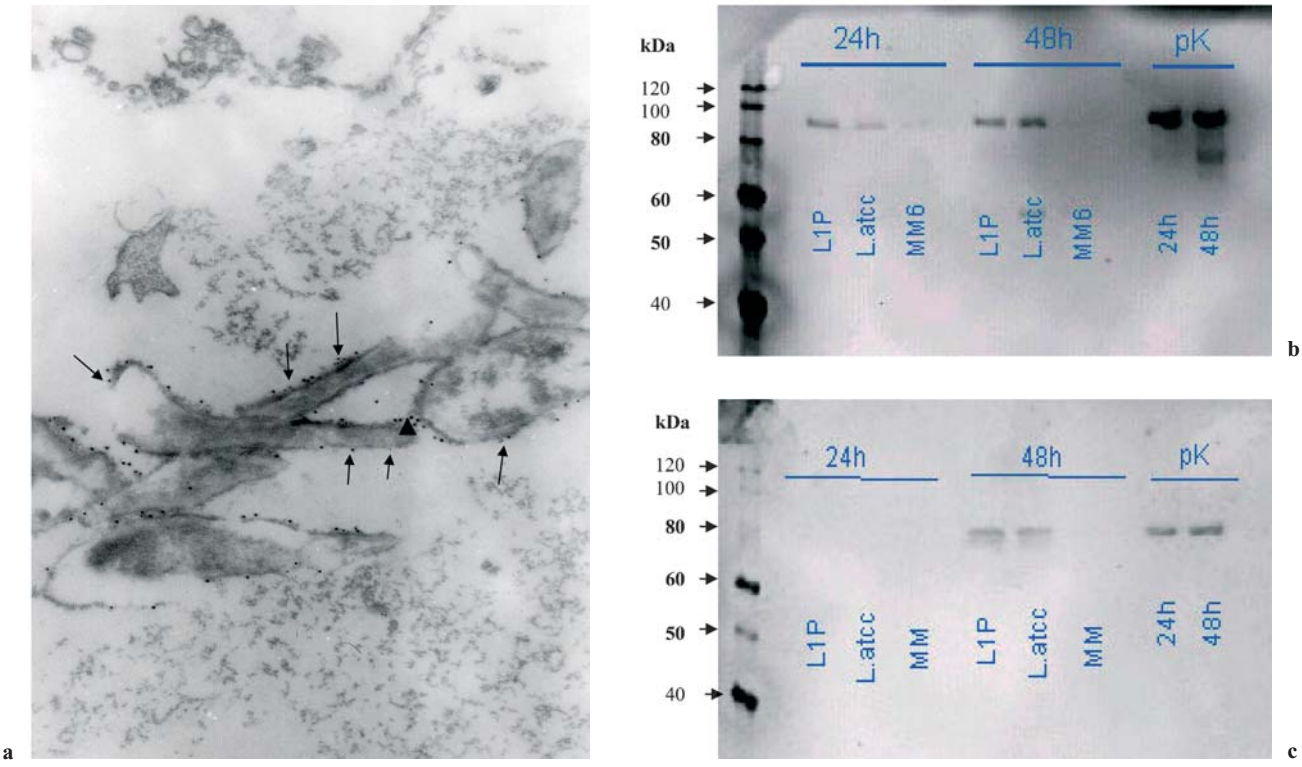

Figure 2. Intracellular adsorption of ER-resident proteins to *L. pneumophila*. MM6 were infected using a bacterium to cell ratio of 10:1. Binding of calnexin to the surface of intracellular *L. pneumophila* within MM6 is visualized by electron microscopy 6 h after infection (*a*). Ten-nanometer gold conjugates identify *L. pneumophila* LPS (arrowheads), 5-nm gold conjugate stain adsorbed calnexin (arrows). Western blot analysis was performed 24 h and 48 h after infection to show adsorption of calnexin (*b*) and BiP (*c*) to *L. pneumophila*. Representatives of four independent experiments are shown. L1P, *L. pneumophila* Sg 1 Pontiac; L.atcc, *L. pneumophila* Sg 1 ATCC 33152 Philadelphia 1; MM6, negative control (immunobeads incubated with noninfected MM6); PK, positive control (lysed MM6).

Intracellular adsorption of ER-resident chaperons to *L. pneumophila*

Binding of calnexin to the surface of the highly replicative intracellular *L. pneumophila* Pontiac within MM6 could be visualized by electron microscopy (fig. 2a). Western blot analysis revealed that 24 h after infection, adsorption of calnexin to *L. pneumophila* was more pronounced in the Pontiac strain than in the agar-adapted Philadelphia strain, whereas adsorption of BiP could not be demonstrated at this time point. After 48 h, the Philadelphia strain acquired a higher amount of calnexin than the Pontiac strain and adsorption of BiP could be demonstrated for both strains (fig. 2b, c). Tapasin and β_2-microglobulin, molecules also involved in the formation of MHC complexes, were not absorbed to the bacterial surfaces (data not shown).

Infection of MM6 cells with *Legionella* species reduces MHC class I expression of host cells, dependent on the bacterial intracellular replication rate

Already 24 h after infection with *L. pneumophila*, MHC class I protein surface expression of MM6 evaluated as percentage of fluorescence-positive cells was reduced around 25%, and after 48 h, the reduction was 50%. The nonreplicative *Legionella* species only marginally reduced MHC class I expression (fig. 3a). Evaluated as mean fluorescence intensity of the single cell, reduction of MHC class I expression was small 24 h after infection, but after 48 h, it was reduced around 80% for *L. pneumophila* and around 30% for the non-*pneumophila* species (fig. 3b). Infection of PBMCs with *Legionella* species showed the same degree and identical kinetics of inhibition of MHC class I expression as was observed in MM6 (data not shown). Addition of 1.5×10^4 U/ml IFN-γ could delay the reduction of MHC class I expression induced by *L. pneumophila* only during the first 12 h of intracellular replication within MM6 (fig. 4). Expression of MHC class II was not influenced by infection with *Legionella* species (data not shown).

Infection with *L. pneumophila* impairs the stimulation of allogeneic PBMCs

Allogeneic stimulation was performed by coincubation of irradiated PBMC 1 with nonirradiated PBMC 2 and determination of IL-2 secretion during coculture. The ability of *L. pneumophila*-infected PBMCs to stimulate IL-2 secretion of allogeneic PBMCs was severely reduced. Whereas a marginal IL-2 secretion could be still observed

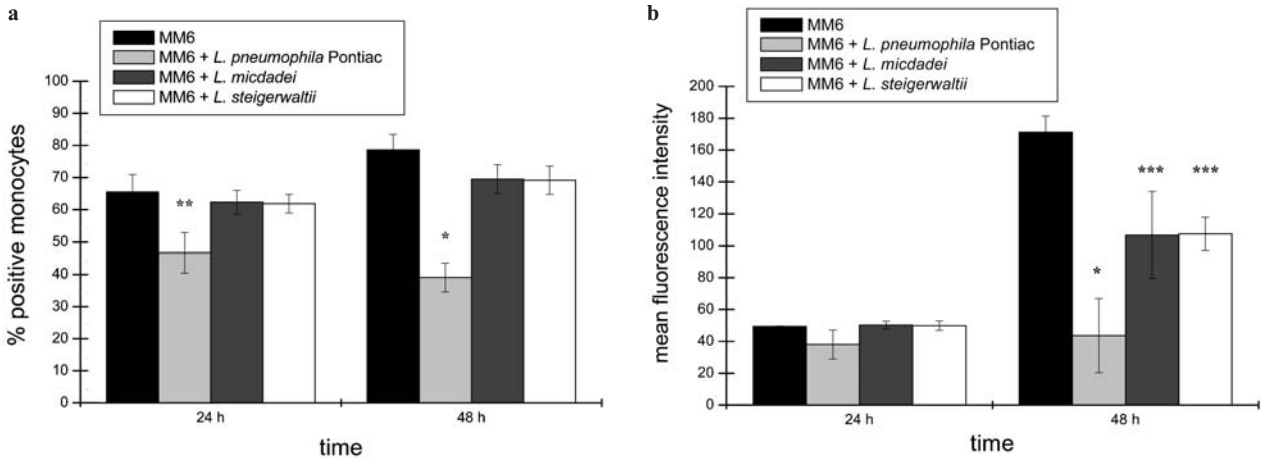

Figure 3. Infection of MM6 with *Legionella* species reduces MHC class I expression of host cells, dependent on the bacterial intracellular replication rate. MM6 were infected using a bacterium to cell ratio of 10:1. After phagocytosis and killing of extracellular bacteria by gentamicin, cells were incubated for 48 h and expression of MHC class I proteins was analyzed by FACS at the indicated time points. Results are expressed as percent fluorescence-positive cells (means ± SD) (*a*) and mean fluorescence intensity (means ± SD) (*b*) from three independent infections. * $p < 10^{-4}$, ** $p < 0.0012$, *** $p < 0.004$.

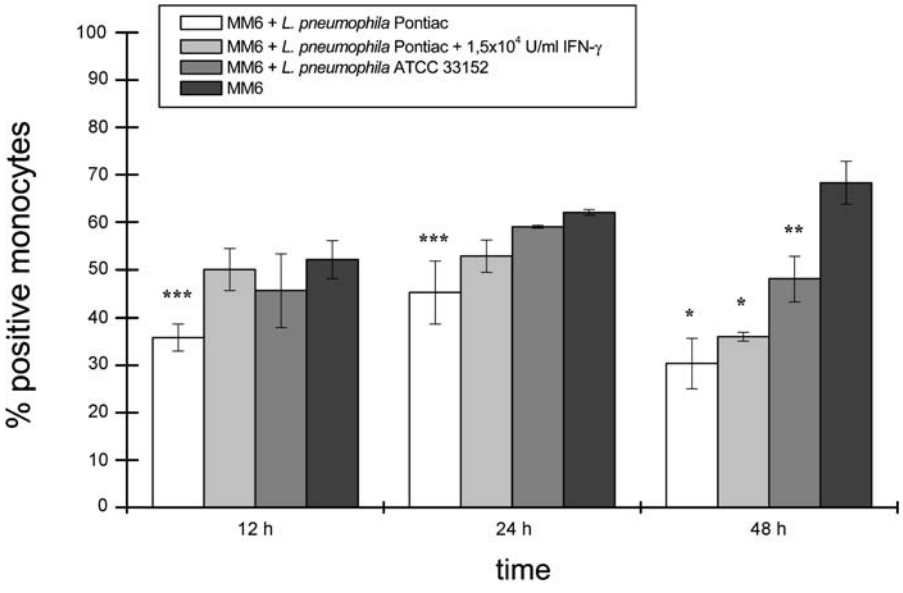

Figure 4. Addition of IFN-γ could delay the reduction of MHC class I expression induced by *L. pneumophila* during the first 12 h of intracellular replication in MM6. Host cells were infected using a bacterium to cell ratio of 10:1 as described. Infection with *L. pneumophila* Pontiac was performed with and without addition of 1.5×10^4 U/ml IFN-γ immediately after phagocytosis and killing of extracellular bacteria by gentamicin. Infected host cells were incubated for 48 h and expression of MHC class I proteins was analyzed by FACS at the indicated time points. Results are expressed as % fluorescence-positive cells (means ± SD) from three independent infections. * $p < 10^{-4}$, ** $p < 0.0004$, *** $p < 0.0006$.

with infected PBMCs 24 h after infection (fig. 5a), there was no IL-2 signal 48 h after infection (fig. 5b) where MHC class I expression was maximally reduced (fig. 3 for comparison).

Autologous NK cells are activated after coincubation with autologous *L. pneumophila*-infected monocytes but are not able to lyse the target cells

After coincubation with *L. pneumophila*-infected autologous monocytes, NK cells were stimulated to produce IFN-γ (fig. 6). IFN-γ production could be correlated to a high intracellular multiplication rate, high MOI and long time after infection-conditions associated with reduced expression of MHC class I proteins (fig. 3 for comparison). Monocytes infected with *L. pneumophila* using a MOI of 10:1 and an infection time of 48 h were able to stimulate autologous NK cells to an extent comparable to K-562 cells, whereas *L. pneumophila*-infected monocytes using an MOI of 1:1 and an infection time of 24 h or monocytes infected with the nonreplicative *L. steiger-*

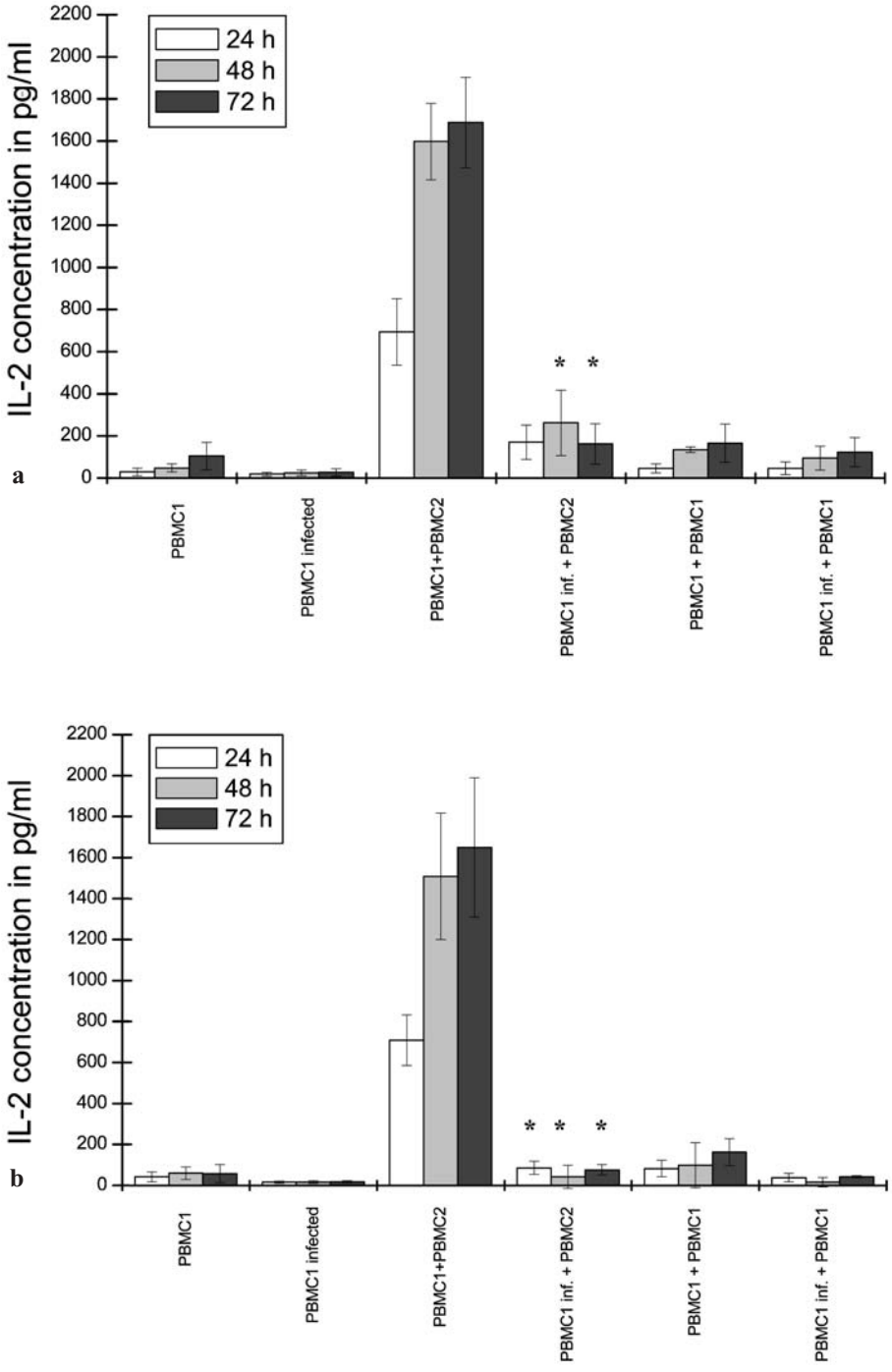

Figure 5. Infection with *L. pneumophila* impairs the stimulation of allogeneic PBMCs. Allogeneic stimulation was performed by coincubation of irradiated PBMC1 with nonirradiated PBMC2 and determination of IL-2 secretion after 24 h, 48 and 72 h of coincubation. To determine the effect of infection with *L. pneumophila*, PBMC1 were used 24 h (*a*) and 48 h (*b*) after infection. PBMC1, PBMC1 uninfected and irradiated (negative control); PBMC1 infected: PBMC1 infected and irradiated (negative control); PBMC1+PBMC2: coincubation of uninfected and irradiated PBMC1 with uninfected and non-irradiated PBMC2 (positive control: allogeneic stimulation); PBMC1 inf. + PBMC2: coincubation of infected and irradiated PBMC1 with uninfected and non-irradiated PBMC2 (impairment of allogeneic stimulation by infection with *L. pneumophila*); PBMC1 + PBMC1: coincubation of uninfected and irradiated PBMC1 with uninfected and non-irradiated PBMC1 (control); PBMC1 inf. + PBMC1: coincubation of infected and irradiated PBMC1 with uninfected and non-irradiated PBMC1 (control). Results are expressed as means ± SD of IL-2 secretion from six independent infections. * $p < 10^{-4}$.

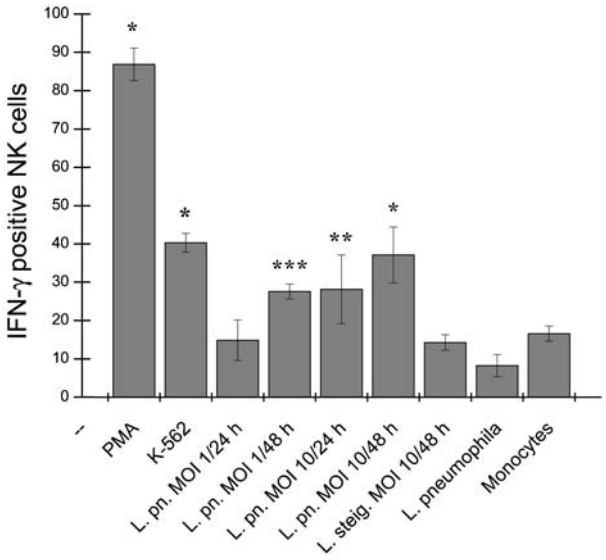

Figure 6. Autologous NK cells are activated after coincubation with autologous *L. pneumophila*-infected monocytes. Monocytes were infected with *L. pneumophila* or *L. steigerwaltii* using an MOI of 10:1 or 1:1 and were incubated for 24 and 48 h. Thereafter, monocytes were irradiated with 30 Gy and were coincubated with IL-2-activated autologous NK cells. Noninfected monocytes served as negative control (monocytes); for control of maximal stimulation, phorbol myristate acetate plus ionomycin (PMA) and K562 cells (K-562) were used. NK cells were measured for intracellular IFN-γ by FACS. Results are expressed as percent IFN-γ positive cells ± SD from three independent infections. L. pn.: *L. pneumophila;* MOI, Multiplicity of infection (1:1, 10:1); 24 h, 48 h: time after infection of monocytes. * $p < 10^{-4}$, ** $p < 0.004$, *** $p < 0.006$.

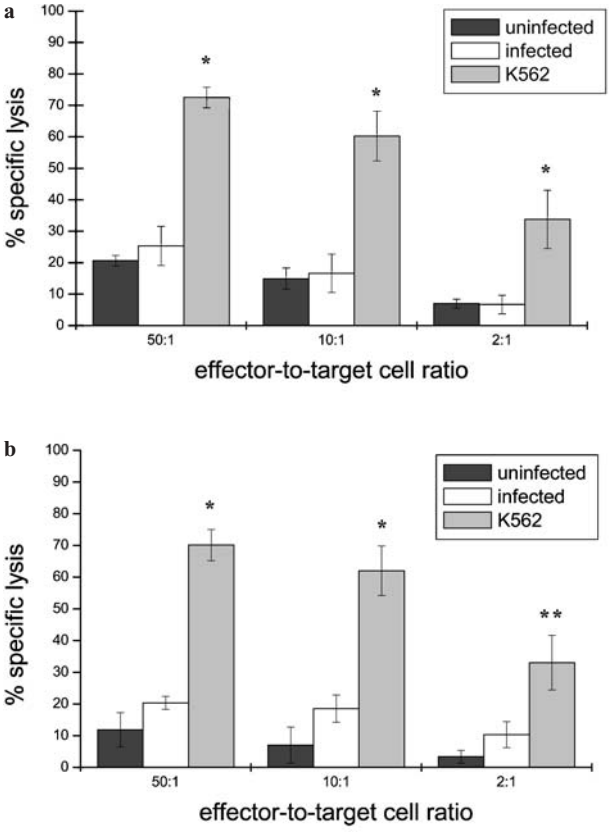

Figure 7. Impaired lysis of autologous, *L. pneumophila*-infected monocytes by NK cells. Monocytes (target cells) infected with *L. pneumophila* using an MOI of 1:1 (*a*) or 10:1 (*b*) were incubated for 24 h and then labeled with $Na_2^{51}CrO_4$ (white bars). IL-2-activated autologous NK cells (effector cells) were added to provide an effector to target cell ratio of 50:1, 10:1 or 2:1. K562 cells served as a positive control (light-gray bars), uninfected autologous monocytes served as negative control (dark-gray bars). Results are expressed as percent specific lysis ± SD of three independent experiments. * $p < 10^{-4}$, ** $p < 0.0005$.

waltii did not show stimulation. Despite the obvious recognition of *L. pneumophila*-infected autologous monocytes, NK cells were not able to lyse these cells efficiently. Whereas incubation of NK cells with K-562 cells resulted in a specific lysis between 40 and nearly 80% depending on the effector-to-target ratio, autologous monocytic cells infected with *L. pneumophila* using an MOI of 1:1 (fig. 7a) or 10:1 (fig. 7b) either showed only marginal or no lysis at all.

Discussion

Interference with MHC antigen presentation pathways is an effective strategy for pathogen evasion of an immune response. The classical paradigm of antigen presentation is that MHC class I molecules bind peptides in the ER that are produced by proteasome-mediated catabolism of endogenous or cytosolic antigens and are transported into the lumen of the ER via the peptide transporter TAP. These antigens are presented to cytolytic $CD8^+$ T cells (CTLs). In contrast, exogenous antigens are phagocytosed and degraded in acidified phagolysosomal vacuoles to yield peptides that bind to MHC class II molecules and are thereby presented to $CD4^+$ T cells. Consequently, viral strategies to disturb antigen processing and presentation include interference with class I molecule assembly and export resulting in down-regulation of MHC class I molecules, combined with strategies to avoid NK cell recognition [reviewed in ref. 30]. On the other hand, it has long been accepted that bacteria residing in endosomal compartments have the advantage of physical sequestration from MHC class I presentation and thereby reduce stimulation of cell-mediated immune responses. Nevertheless, a number of studies have demonstrated that exogenous antigens with no intrinsic ability to escape from phagosomes into the cytosol are nonetheless capable of generating MHC class I-restricted immune responses. Protective CTL responses have been generated against vacuolar pathogens such as noncytosolic *Listeria monocytogenes*, *Mycobacterium* and *Leishmania* [31–35]. In addition, interference with MHC class I expression has

been documented. *Chlamydia pneumoniae* down-regulates MHC class I expression but not MHC class II expression by induction of IL-10 synthesis [36] and by degradation of RFX5 [37]. Infection of bovine macrophages with *Mycobacterium avium* promoted the down-regulation of MHC class I and class II molecules depending on the virulence of a given subspecies. The suppressive factor was found to be a constituent of the cell wall. Down-regulation of MHC class I expression resulted in a diminished capacity of primed autologous lymphocytes to lyse *M. avium*-infected macrophages [38]. The vacuoles that harbor *L. pneumophila* resemble those of mycobacteria or chlamydiae in many aspects, in particular with regard to the blocked maturation of the phagosome [39, 40]. By using four *Legionella* strains differing in their ability to multiply within human monocytes, we could show that a high intracellular replication rate was associated with a more pronounced reduction of MHC class I expression on the surface of infected host cells. In addition, we could demonstrate that the extent of intracellular adsorption of ER-resident chaperons to the bacteria correlated with their intracellular replication rate, in particular during the first 24 h of intracellular replication. Adsorption of these chaperons was a specific event since other molecules involved in the formation of MHC complexes such as tapasin or β_2-microglobulin [41] were not adsorbed to the bacterial surfaces (data not shown). Two recent reports have shown that fusion between ER and phagosomes is a source of membrane for phagosome formation in macrophages, and defines an MHC class I cross-presentation compartment [41, 42]. ER proteins such as calnexin were highly enriched in phagosomes, in particular in the earliest compartments. *L. pneumophila* resides within a compartment staining positive for resident proteins of the ER [40, 44, 45]. The morphological similarities between replicative vacuoles harboring *Legionella* and the host ER indicate that these bacteria have developed a way to enter the ER lumen by altering phagosome transport [46]. Within this compartment, they may get access to the ER-resident chaperons. We could show by electron microcopy that calnexin adsorption to the surface of *L. pneumophila* can be detected from 6 h after infection. Thus, ER-resident chaperons may no longer be available to take part in the formation of the MHC class I complex.

Whether *L. pneumophila*-specific CTLs are involved in the lysis of infected monocytes has never been investigated and the peptides presented to CTLs remain to be characterized. The almost complete inhibition of stimulation of allogeneic PBMCs induced by the remarkably reduced expression of MHC class I proteins on the surface of *L. pneumophila*-infected human monocytes may be indicative for an impaired antigen presentation to CTLs. How *L. pneumophila* antigens are presented to CTLs remains to be determined. Several alternative MHC class I pathways to induce CTL responses have been described. These include direct injection of pathogen-derived antigenic material into the cytosol, recycling and endocytosis of class I molecules followed by reassembly with peptide ligand and β_2-microglobulin within the endosome, and regurgitation of peptide antigens from the endosomal compartment onto the cell surface for association with preformed MHC class I molecules [reviewed in refs. 47, 48]. The ability of *L. pneumophila* to insert pores into eukaryotic membranes [49] and the presence of a type IV secretion system [50] may facilitate the delivery of *Legionella* antigens into the cytosol. Furthermore, peptides can be retranslocated from the phagosome to the cytoplasmic side of phagosomes by the Sec16/chaperone retranslocation machinery, have access to the ubiquitin/proteasome complex, with resulting peptides being shuttled back into the phagosome by TAP where they are loaded onto MHC class I molecules [42, 43, 51].

Reduction of MHC class I molecules on the surface of *L. pneumophila*-infected human monocytes induced the production of IFN-γ by autologous NK cells. A high intracellular multiplication rate, high MOI and long time after infection – conditions associated with reduced expression of MHC class I proteins – stimulated autologous NK cells to an extent comparable to the positive control using K-562 cells. Surprisingly, these NK cells were not able to lyse *L. pneumophila*-infected autologous monocytes efficiently. The cause for this phenomenon remains unclear. NK cell killing is inhibited primarily by the presence of either HLA-C or HLA-E and to a lesser extent by some HLA-B alleles [52]. HIV-infected cells were recently shown to selectively down-regulate HLA-A and HLA-B while preserving expression of HLA-C and HLA-E and are thereby protected from lysis by NK cells [53]. Since we used a pan-class I antibody for determination of MHC class I expression by FACS, we could not differentiate between the MHC class I isotypes. Thus, induction of IFN-γ production in NK cells indicates that, in vivo, intracellular replication of *L. pneumophila* growth may be restricted by IFN-γ-mediated activation of infected monocytes [17, 19–22] but *L. pneumophila* may escape from CTLs as well as from destruction by NK cells via regulated reduction of MHC class I isotypes on the surface of infected host cells.

Acknowledgement. The authors want to thank Prof. W. Held (Ludwig Institut, Lausanne, Switzerland), Dr. S. Stefanovic and Dr. A. Steinle (Department of Immunology, University of Tuebingen) for helpful discussion and monitoring of our work. The authors are grateful to Dr. M. Topp (Internal Medicine, University of Tuebingen) for technical assistance and to Prof. K. Dietz (Biometry, University of Tuebingen) for statistical support. This work was in part supported by a grant of the Graduate Programme No. 794 of the German Research Foundation (DFG) to B. N.

1 Ciesielski C. A., Blaser M. J. and Wang W. L. (1986) Serogroup specificity of *Legionella pneumophila* is related to lipopolysaccharide characteristics. Infect. Immun. **51:** 397–404
2 Conlan J. W. and Ashworth L. A. (1986) The relationship between the serogroup antigen and lipopolysaccharide of *Legionella pneumophila*. J. Hyg. Lond. **96:** 39–48
3 Weeratna R., Stamler D. A., Edelstein P. H., Ripley M., Marrie T., Hoskin D. et al. (1994) Human and guinea pig immune responses to *Legionella pneumophila* protein antigens OmpS and Hsp60. Infect. Immun. **62:** 3454–3462
4 Horwitz M. A. and Silverstein S. C. (1981) Interaction of the legionnaires' disease bacterium (*Legionella pneumophila*) with human phagocytes. II. Antibody promotes binding of *L. pneumophila* to monocytes but does not inhibit intracellular multiplication. J. Exp. Med. **153:** 398–406
5 Plouffe J. F. and Baird I. M. (1981) Lymphocyte transformation to *Legionella pneumophila*. J. Clin. Lab. Immunol. **5:** 149–152
6 Plouffe J. F. and Baird I. M. (1982) Lymphocyte blastogenic responses to *L. pneumophila* in acute legionellosis. J. Clin. Lab. Immunol. **7:** 43–44
7 Horwitz M. A. (1983) Cell mediated immunity in Legionnaires' disease. J. Clin. Invest. **71:** 1686–1697
8 Friedman H., Widen R., Klein T. and Johnson W. (1983) *Legionella pneumophila* induced immunostimulation and blastogenesis of normal mouse spleen cells in vitro. Proc. Soc. Exp. Biol. Med. **174:** 212–216
9 Friedman H., Widen R., Klein T., Searls L. and Cabrian K. (1984) *Legionella pneumophila* induced blastogenesis of murine lymphoid cells in vitro. Infect. Immun. **43:** 314–319
10 Friedman F., Widen R., Klein T. and Friedman H. (1984) Lymphoid cell blastogenesis as an in vitro indicator of cellular immunity to *Legionella pneumophila* antigens. J. Clin. Microbiol. **19:** 834–837
11 Horwitz M. A. and Silverstein S. C. (1981) Activated human monocytes inhibit the intracellular multiplication of Legionnaires' disease bacteria. J. Exp. Med. **154:** 1618–1635
12 Yamamoto Y., Klein T. W., Newton C. A., Widen R. and Friedman H. (1987) Differential growth of *Legionella pneumophila* in guinea pig versus mouse macrophage cultures. Infect. Immun. **55:** 1369–1374
13 Bhardwaj N., Nash T. W. and Horwitz M. A. (1986) Interferon gamma activated human monocytes inhibit the intracellular multiplication of *Legionella pneumophila*. J. Immunol. **137:** 2662–2669
14 Whitaker-Dowling P., Dowling J. N., Liu L. and Youngner J. S. (1986) Interferon inhibits the growth of *Legionella micdadei* in mouse L cells. J. Interferon Res. **6:** 107–114
15 Jensen W. A., Rose R. M., Wasserman A. S., Kalb T. H., Anton K. and Remold H. G. (1987) In vitro activation of the antibacterial activity of human pulmonary macrophages by recombinant gamma interferon. J. Infect. Dis. **155:** 574–577
16 Nash T. W., Libby D. M. and Horwitz M. A. (1988) IFN gamma activated human alveolar macrophages inhibit the intracellular multiplication of *Legionella pneumophila.* J. Immunol. **140:** 3978–3981
17 Byrd T. F. and Horwitz M. A. (1989) Interferon gamma activated human monocytes downregulate transferrin receptors and inhibit the intracellular multiplication of *Legionella pneumophila* by limiting the availability of iron. J. Clin. Invest. **83:** 1457–1465
18 Skerrett S. J. and Martin T. R. (1992) Recombinant murine interferon gamma reversibly activates rat alveolar macrophages to kill *Legionella pneumophila*. J. Infect. Dis. **166:** 1354–1361
19 Byrd T. F. and Horwitz M. A. (1990) Interferon gamma aktivated human monocytes downregulate the intracellular concentration of ferritin: a potential new mechanism for limiting iron availability to *L. pneumophila* and subsequently inhibiting intracellular multiplication. Clin. Res. **38:** 481A
20 Gebran S. J., Newton C., Yamamoto Y., Widen R., Klein T. W. and Friedman H. (1994): Macrophage permissiveness for *Legionella pneumophila* growth modulated by iron. Infect. Immun. **62:** 564–568
21 Gebran S. J., Yamamoto Y., Newton C., Klein T. W. and Friedman H. (1994) Inhibition of *Legionella pneumophila* growth by gamma interferon in permissive A/J mouse macrophages: role of reactive oxygen species, nitric oxide, tryptophan, and iron(III). Infect. Immun. **62:** 3197–3205
22 Gebran S. J., Yamamoto Y., McHugh S., Newton C., Klein T. W. and Friedman H. (1994) Differences and similarities in permissive A/J versus non permissive BALB/c murine macrophages infected with *Legionella pneumophila*: the role of iron. FEMS Immunol. Med. Microbiol. **9:** 7–14
23 Blanchard D. K., Stewart W. E., Klein T. W., Friedman H. and Djeu J. Y. (1987) Cytolytic activity of human peripheral blood leukocytes against *Legionella pneumophila* infected monocytes: characterization of the effector cell and augmentation by interleukin 2. J. Immunol. **139:** 551–556
24 Blanchard D. K., Friedman H., Stewart W. E., Klein T. W. and Djeu J. Y. (1988) Role of gamma interferon in induction of natural killer activity by *Legionella pneumophila* in vitro and in an experimental murine infection model. Infect. Immun. **56:** 1187–1193
25 Resnick M., Roguel N., Bercovier H., Enk C., Frankenburg S. and Kedar E. (1988) Lysis of murine macrophages infected with intracellular pathogens by interleukin 2 activated killer (LAK) cells in vitro. Cell. Immunol. **113:** 214–219
26 Gerhardt H., Walz M. J., Faigle M., Northoff H., Wolburg H. and Neumeister, B. (2000) Localization of *Legionella* bacteria within ribosome-studded phagosomes is not restricted to *Legionella pneumophila*. FEMS Microbiology. Lett. **192:** 145–152
27 Neumeister B., Faigle M., Sommer M., Zähringer U., Stelter F., Witt S. et al. (1998) Low endotoxic potential of *Legionella pneumophila* lipopolysaccharide due to failure of interaction with the monocytic LPS receptor CD14. Infect. Immun. **66:** 4151–4157
28 Neumeister B., Schöniger S., Faigle M., Eichner M. and Dietz K (1997) Multiplication of different *Legionella* species in Mono Mac 6 cells and in *Acanthamoeba castellani*. Appl. Environ. Microbiol. **63:** 1219–1224
29 Sambrock J. and Russell D. W. (2001) Molecular Cloning: A Laboratory Manual. Cold Spring Harbour Laboratory Press, Cold Spring Harbor, N. Y.
30 Brodsky F. M., Lem L., Solache A. and Bennett E. M. (1999) Human pathogen subversion of antigen presentation. Immunol. Rev. **168:** 199–215
31 Zwickes H. L. and Potter T. A. (1999) Antigen secreted from noncytosolic *Listeria monocytogenes* is processed by the classical MHC class I processing pathway. J. Immunol. **162:** 6341–6350
32 Canaday D. H., Ziebold C., Noss E. H., Chervenak K. A., Harding C. V. and Boom W. H. (1999) Activation of human $CD8^+$ $\alpha\beta$ TCR^+ cells by *Mycobacterium tuberculosis* via an alternate class I MHC antigen-processing pathway. J. Immunol. **162:** 372–379
33 Kaleab B., Ottenoff T., Converse P., Halapi E., Tadesse G., Rottenberg M. et al. (1990) Mycobacterial-induced cytotoxic T cells as well as nonspecific killer cells derived from healthy individuals and leprosy patients. Eur. J. Immunol. **20:** 2651–2659
34 Flynn J. L., Goldstein M. M., Triebold K. J., Koller B. and Bloom B. R. (1992) Major histocompatibility complex class I-restricted T cells are required for resistance to *Mycobacterium tuberculosis* infection. Proc. Natl. Acad. Sci. USA **89:** 12013–12017
35 Conceicao-Silva F. da, Perlaza B. L., Louis J. A. and Romero P. (1994) *Leishmania major* infection in mice primes for specific

major histocompatibility complex class I-restricted CD8+ cytotoxic T cell responses. Eur. J. Immunol. **24:** 2813–2817

36 Caspar-Bauguil S., Puissant B., Nazzal D., Lefevre J. C., Thomsen M., Salvayre R. et al. (2000) *Chlamydia pneumoniae* induces interleukin-10 production that down-regulates major histocompatibility complex class I expression. J. Infect. Dis. **182:** 1394–1401

37 Zhong G., Liu L., Fan T., Fan P. and Ji H. (2000) Degradation of transcription factor RFX5 during the inhibition of both constitutive and interferon gamma-inducible major histocompatibility complex class I expression in *Chlamydia*-infected cells. J. Exp. Med. **191:** 1525–1534.

38 Weiss D. J., Evanson O. A., McClenahan D. J., Abrahamsen M. S. and Walcheck B. K. (2001) Regulation of expression of major histocompatibility antigens by bovine macrophages infected with *Mycobacterium avium* subsp. *paratuberculosis* or *Mycobacterium avium* subsp. *avium*. Infect. Immun. **69:** 1002–1008

39 Horwitz M. A. (1983) The Legionnaires' disease bacterium (*Legionella pneumophila*) inhibits phagosome-lysosome fusion in human monocytes. J. Exp. Med. **158:** 2108–2126

40 Swanson M. S. and Isberg R. R. (1995) Association of *Legionella pneumophila* with the macrophage endoplasmic reticulum. Infect. Immun. **63:** 3609–3620

41 Sadasivan B., Lehner P. J., Ortmann B., Spies T. and Cresswell P. (1996) Roles for calreticulin and a novel glycoprotein, tapasin, in the interaction of MHC class I molecules with TAP. Immunity **5:** 103–114

42 Gagnon E., Duclos S., Rondeau C., Chevet E., Cameron P. H., Steele-Mortimer O. et al. (2002) Endoplasmic reticulum-mediated phagocytosis is a mechanism of entry into macrophages. Cell **110:** 119–131

43 Guermonprez P., Saveanu L., Kleijmeer M., Davoust J., Van-Endert P. and Amigorena S. (2003) ER-phagosome fusion defines an MHC class I cross-presentation compartment in dendritic cells. Nature **425:** 397–402

44 Kagan J. C. and Roy C. R. (2002) *Legionella* phagosomes intercept vesicular traffic from endoplasmic reticulum exit sites. Nat. Cell Biol. **4:** 945–954

45 Derré I. and Isberg R. R. (2004) *Legionella pneumophila* replication vacuole formation involves rapid recruitment of proteins of the early secretory system. Infect. Immun. **72:** 3048–3053

46 Roy C. R. and Tilney L. G. (2002) The road less traveled: transport of *Legionella* to the endoplamic reticulum. J. Cell Biol. **158:** 415–419

47 Heath W. R. and Carbone F. R. (2001) Cross-presentation in viral immunity and self-tolerance. Nat. Rev. Immunol. **1:** 126–135

48 Maksymowych W. P. and Kane K. P. (2000) Bacterial modulation of antigen processing and presentation. Microb. Infect. **2:** 199–211

49 Kirby J. E., Vogel J. P., Andrews H. L. and Isberg R. R. (1998) Evidence for pore-forming ability by *Legionella pneumophila*. Mol. Microbiol. **27:** 323–336

50 Segal G., Russo J. J. and Shuman H. A. (1999) Relationships between a new type IV secretion system and the icm/dot virulence system of *Legionella pneumophila*. Mol. Microbiol. **34**: 799–809

51 Houde M., Bertholet S., Gagnon E., Brunet S., Goyette G., Laplante A. et al. (2003) Phagosomes are competent organelles for antigen cross-presentation. Nature **425:** 402–406

52 Lanier L. L. (1998) NK cell receptors. Annu. Rev. Immunol. **16:** 359–393

53 Cohen G. B., Gandhi R. T., Davis D. M., Mandelboim O., Chen B. K., Strominger J. L. et al. (1999) The selective downregulation of class I major histocompatibility complex proteins by HIV–1 protects HIV-infected cells from NK cells. Immunity **10:** 661–671

54 Clemens D. L. and Horwitz M. A. (1992): Membrane sorting during phagocytosis: selective exclusion of major histocompatibility complex molecules but not complement receptor CR3 during conventional and coiling phagocytosis. J. Exp. Med. **175:** 1317–1326