INFECTIONS AND ATHEROSCLEROSIS: *CHLAMYDOPHILA PNEUMONIAE* PERSISTENCE IN MICE, INTERACTION OF *CHLAMYDOPHILA PNEUMONIAE* AND HUMAN CYTOMEGALOVIRUS WITH HUMAN DENDRITIC CELLS

Ph. D. Thesis

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Publications related to the Thesis

I. Publications incorporated in the Thesis:

I.1. **Kis Z**, Burian K, Treso B, Acs K, Prohaszka Z, Fust G, Gonczol E, Endresz V. Inflammatory- and immune responses in relation with bacterial replication in mice following re-infections with *Chlamydophila pneumoniae*. Inflamm Res, accepted. **IF.: 1.485**

I.2. **Kis Z**, Treso B, Burian K, Endresz V, Pallinger E, Nagy A, Toth A, Takacs M, Falus A, Gonczol E. Expression of bacterial genes and induction of interferon-gamma in human myeloid dendritic cells during persistent infection with *Chlamydophila pneumoniae*. FEMS Immunol Med Microbiol, accepted. **IF.: 2.281**

I.3. **Kis Z**, Pallinger E, Endresz V, Burian K, Falus A, Berencsi G, Gonczol E. A soluble factor(s) released by MRC-5 cells early and late after human cytomegalovirus infection induces maturation of monocyte-derived dendritic cells. Arch Virol 2006; 151: 2277-87. **IF.: 1.850**

II. Further publications related to the thesis:

II.1. Petrovay F, Heltai K, **Kis Z**, Treso B, Gonczol E, Burian K, Endresz V, Valyi-Nagy I. Chronic infection and histamine, CRP and IL-6 level after percutaneous transluminal coronary angioplasty. Inflamm Res 2007; 56: 362-7. **IF.: 1.485**

II.2. **Kis Z,** Sas K, Gyulai Z, Treso B, Petrovay F, Kapusinszky B, Csire M, Endresz V, Burian K, Mandi Y, Vecsey L, Gonczol E. Chronic infections and genetic factors in the development of ischemic stroke. New Microbiol 2007; 30: 213-20. **IF.: 0.806**

II.3. Virok D, **Kis Z**, Kari L, Barzo P, Sipka R, Burian K, Nelson DE, Jackel M, Kerenyi T, Bodosi M, Gonczol E, Endresz V. *Chlamydophila pneumoniae* and human cytomegalovirus in atherosclerotic carotid plaques combined presence and possible interactions. Acta Microbiol Immunol Hung 2006; 53: 35-50.

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III.1. Treso B, **Kis Z**, Toth A, Endresz V, Burian K, Takacs M, Gonczol E. Comparison of bacterial gene expression and production of infectious particles in HEp-2 cells treated with penicillin and infected either with a respiratory or a vascular strain of *Chlamydophila pneumoniae*. 15th International Congress of the Hungarian Society for Microbiology. Budapest, 18-20 Jul., 2007.

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Abbreviation:

APC	allophycocyanine
AT	atherosclerosis
BrdU	Bromodeoxyuridine
Cpn	*Chlamydophila pneumoniae*
CTL	cytotoxic T lymphocyte
DCs	dendritic cells
DC-SIGN	DC-specific ICAM-3-grabbing nonintegrin
DC-SIGNR	DC-specific ICAM-3-grabbing nonintegrin receptor
dIF	direct immunofluorescence
DNA	deoxyribonucleic acid
EB	elementary body
EBV	Epstein-Barr virus
FITC	fluorescein isothiocyanate
GM	geometric mean
GM-CSF	granulocyte-monocyte colony stimulating factor
HCMV	human cytomegalovirus
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HRP	horse-radish peroxidase
hsp	heat shock protein
HSV	herpes simplex virus
iDC	immature dendritic cells
IFN	interferon
IFU	inclusion forming unit
Ig	immunoglobulin
iIF	indirect immunofluorescence
IL	interleukin
kDa	kilodalton
LCs	Langerhans dendritic cells
LPS	lipopolysaccharide
MHC	major histocompatibility complex
MIE	major immediate early
MIEP	major immediate early promoter
MOI	multiplicity of infection
nPCR	nested polymerase chain reaction
PBMCs	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PE	phycoerythrin
PerCP	peridinin-chlorophyll-protein
SD	standard deviation
TGF	transforming growth factor
TLRs	Toll-like receptors
TNF	tumor necrosis factor

1. Summary

We investigated the characteristics of acute and chronic infections with *Chlamydophila pneumoniae* (*Cpn*) in mice. BALB/c mice were repeatedly infected with the bacterium and tested during a 1-year period. After primary infection culture positivity or the presence of *Cpn* DNA in the lung of all mice was observed for a period of 14 or 28 days, respectively. In the blood *Cpn* DNA was detected up to 14 days after infection. After re-infections, culture positivity was detected only for 2 days. The persistence of DNA in lungs and blood was shorter (14 days and 2 days). Detection of DNA at late time points after primary or re-infections (10, 18, 49 weeks) indicated persistent state in a few mice. These results provide an evidence for chronic infection after primary or re-infection and promote an understanding of the patho- and immune-mechanisms after *Cpn* re-infections in humans.

The link between human DCs present in atherosclerotic lesion and certain pathogens, possibly associated with the development of atheromas, is not fully clarified. We examined the interactions of human monocyte-derived immature dendritic cells (iDCs) with *Cpn* and human cytomegalovirus (HCMV). The exponential production of *Cpn* infectious elementary bodies in DCs was not observed. *Cpn* infection induced the maturation and functional activation of DCs, and *Cpn* antigens were present in all of the subpopulations during the maturation process. Chlamydial transcripts of the *16S rRNA*, *groEL-1* and *omcB* genes were expressed, as determined by quantitative real-time PCR, but expression of the *ftsK* gene was limited. DC cultures produced interferon-gamma (IFN-γ), but the presence of IFN-γ in the culture medium was not the major factor that limited the growth of *Cpn*, as was shown by neutralization of the IFN-γ. A cell population identified as producing IFN-γ had no markers for T, B, natural killer, monocyte cells or macrophages, but displayed DC morphology and the expression of specific DC markers, such as CD11c and HLA-DR. These results reveal a persistent *Cpn* infection of DCs with the expression of some, but not cell division-related genes and the production of IFN-γ.

A HCMV strain passaged 10 times in MRC-5 human fibroblast cells failed to express immediate early antigens in iDCs after infection. However, both the early and the late HCMV conditioning medium, harvested from MRC-5 cells at 24 hours or 7-9 days after infection, respectively, induced higher ratio of DCs expressing maturation markers (CD40, CD83, CD86 and HLA-DR) on the surface of the cells. HCMV conditioning medium ultracentrifuged to remove virus particles exhibited a similarly enhanced expression of DC maturation markers. DCs treated with HCMV conditioning medium harvested late after

infection increased the percentages of IFN-γ producing autologous CD4+ and CD8+ cells of seropositive donors and stimulated HCMV-specific lymphoproliferative responses. The early HCMV conditioning medium was also able to induce the functional maturation of DCs, as demonstrated by supplementing this medium with a *Cpn* antigen that we have selected as a foreign antigen. Our results contribute to our better understanding of the complex interactions between DCs and the investigated pathogens, and may promote clarification of the mechanisms of immune and inflammatory reactions to acute and chronic *Cpn* and HCMV infections.

2. Introduction

The inability of traditional risk factors such as hypercholesterolemia, hypertension and smoking to explain the incidence of atherosclerosis (AT) in about 50% of the cases prompted a search for additional risk factors involved in the development of the disease. Infectious agents have long been suspected to initiate/contribute to the process of AT (118, 73, 88). It has also been suggested that inflammation, either related to infectious agents or independent from infection, may mediate the atherogenic process (16, 99, 104). Genetic variations of certain host genes or their promoters, such as interleukin (IL) -8, CD14, IL-6, P-selectin and cathepsin G, may also influence the immune response to the pathogens or to other inflammatory stimuli (2, 5, 65).

The involvement of infectious agents in the process of human AT was suggested by the detection of the presence of various infectious agents in human AT lesions (27, 68, 70, 98, 119, 121), by the seroepidemiological data indicating higher rates of seropositivity to these agents in AT patients than in control populations (20, 37, 33, 65, 105, 107) and by the animal studies in which AT lesions similar to human AT lesions were induced in animals infected with human pathogens (12, 21, 53). The most strongly implicated infectious agents are *Chlamydophila pneumoniae* (*Cpn*), an obligate intracellular bacterium, and human cytomegalovirus (HCMV), an ubiquitous herpesvirus, both infecting humans early in life and establishing latency in the infected host. Other pathogens, such as *Helicobacter pylori*, herpes simplex virus (HSV), and certain bacteria causing gingivostomatitis, have also been suggested as agents contributing to the development of the disease (28, 30, 80, 86). It is noteworthy that, whereas *Cpn* was not present (or only very rarely) in non-atheromatous tissues, HCMV and HSV DNA were also detected in normal areas of the vessel walls (52, 70, 81). Interestingly, *Cpn* was cultured from the coronary arteries of a patient with coronary AT, indicating the presence of live bacteria in the diseased tissues (98). In our study, mouse infected with mouse cytomegalovirus and superinfected with *Cpn* showed histopathological markers of aorta thickening (21). In our other study, the presence of both *Cpn* and HCMV DNA was demonstrated in some human carotid plaques (121). The co-incidence of *Cpn* and HCMV was higher in patients with acute coronary syndromes than in controls as it was revealed by histochemistry (74). These data indicate that double infection with these two pathogens represent a higher risk for the development of AT than infection with either of these pathogens. Some seroepidemiological data have also suggested that HCMV plays a role in the pathogenesis of restenosis after coronary intervention (131).

The cells in the AT lesions that harbor the DNA of these pathogens have not been clearly identified. It is possible that the pathogens infect endothelial and smooth muscle cells of the arterial wall and reside in these cells in a non-replicating form, but these pathogens may well also be present in the monocyte/macrophages and lymphocytes that are components of the AT lesions. Dendritic cells (DCs) deserve special attention, since AT plaques are rich in DCs (14, 15, 76, 124).

However, some reports not confirming an association between AT and infection with *Cpn* or HCMV should also be considered. These results do not find higher rate of the detection of pathogen DNA in atheromatous lesions than in control areas of the vessels (38, 43, 89), or higher antibody levels to the implicated pathogens (61), or to other pathogens in AT patients than in control individuals (125). The negative data may indicate the complex pathomechanism and multifactorial nature (infections, genetic factors, immune responses) of AT. The negative data may also be explained by high rate of infection with *Cpn* and HCMV in certain populations, including the control subjects, and by the not-standardized techniques for the detection of the pathogens.

2.1. *Chlamydophila pneumoniae*

Chlamydiaceae are obligate intracellular bacteria and several species, such as *Chlamydia trachomatis*, *Chlamydophila psittaci,* and *Chlamydophila pneumoniae* have the ability to infect humans. They cause several forms of infection, ranging from asymptomatic infection to infertility, blindness or pneumonia.

Like all chlamydiae, *Cpn* undergoes a developmental cycle in which two functionally and morphologically distinct bacterial cell types are recognized. The infectious cell type, which is specialized for extracellular survival and transmission, is termed the elementary body (EB). The intracellular, vegetative cell type is called the reticulate body (RB). The developmental cycle is initiated by endocytosis of an EB by an eukaryotic host cell. Chlamydiae remain within an intracellular vacuole, termed an inclusion, for their entire developmental cycle. Shortly after internalization, EBs begin to reorganize and differentiate into RBs, which then begin to multiply by binary fission. Late in the cycle, logarithmic growth ceases as RBs commence to reorganize into EBs, which are released upon lysis of the host cells (103).

Cpn is known to cause a large spectrum of symptoms in the respiratory tract, ranging from an asymptomatic „carriage" state to severe forms of community-acquired pneumonia. *Cpn*-specific antibodies and T cell-mediated immunity develop following *Cpn* infection, but

full protection is not exerted (47). The prevalence of antibodies to *Cpn* increases from the age of 5 years to 50% by the age of 20 years, and slowly continues to increase among adults, indicating that re-infections are common (69).

Cpn also has a tendency to cause persistent infection (39). Chlamydial persistence has been described as a viable but noncultivable growth stage resulting in a long-term relationship with the infected host cell (8). *In vitro* persistence models have been developed by treating the infected susceptible cells with interferon gamma (IFN-γ), or various antibiotics or by withdrawal of important nutritions from the culture medium (54). These studies suggested alterations in the morphology of chlamydial inclusions (small inclusions containing fewer, but larger RB) and upregulation or downregulation of certain chlamydial genes involved either in DNA replication or cell division during persistence. However, despite the general similarities, significant differences in growth and ultrastructural characteristics have also been reported among different systems or within a given system (54).

Several lines of evidence suggest that persistent *Cpn* infections are involved in the pathogenesis of certain chronic diseases, including atherosclerosis (24), asthma (44) and neuronal diseases such as late-onset Alzheimer's disease (7). Infection of non-respiratory sites by *Cpn* requires evasion from the respiratory tract via the bloodstream. *Cpn* DNA has also been detected in the peripheral blood mononuclear cells (PBMCs) of a variable percentage of healthy individuals and in patients with cardiovascular disease (18).

The available data on the characteristics of experimental *in vivo* infections (primary and re-infections) in the animal models are incomplete. In previous studies culturable *Cpn* was demonstrated after primary and secondary infection in BALB/c mouse lungs for 17 and 8 days, respectively (94). *Cpn* DNA in the lungs and in PBMCs was described in other mouse strains (31, 32, 83). However, it was not shown how long after infection or re-infections bacterial DNA was present in the blood and what the copy number of *Cpn* was in the lungs and blood. The long term presence of bacterial compounds, especially proteins in the infected tissues might provide a constant stimulus for the cellular elements of the immune systems, including DCs. Such stimuli might provide an explanation for the association of chronic *Cpn* infection with certain chronic inflammatory diseases, such as atherosclerosis.

2.2. Human cytomegalovirus

The human cytomegalovirus (HCMV) species is a member of the cytomegalovirus genus, betaherpesvirinae subfamily, herpesviridae family that may cause infectious mononucleosis, congenital malformations and complications of organ transplantation. The replication of cytomegaloviruses is strongly species-specific, HCMV infects and replicates only in human cells (84).

HCMV infects the majority of the population (50-90%) with the ability to establish a life-long latency in the infected individuals. HCMV periodically reactivates in the latently infected individuals, causing severe diseases in immunocompromised patients. Early epidemiological studies demonstrated the transmission of HCMV by blood products, bone marrow grafts and solid organs. Studies on the separated peripheral blood cell population derived from individuals with HCMV disease or asymptomatically infected individuals suggested that the myeloid lineage of hematopoetic cells, including myeloid progenitors, monocytes and DCs, is an important reservoir for HCMV (45, 71, 93, 116).

HCMV adsorbs to many cell types in which replication does not occur, owing to the block in the transcription of the major immediate early gene (MIE) which is the first set of viral genes expressed in productive infection and in reactivation (84). Productive HCMV infection and reactivation require an ordered cascade of gene expression and are dependent on the expression of the MIE gene products of the MIE72 and MIE86 proteins (MIE1 and MIE2, respectively). The MIE gene products are nuclear phosphoproteins that play a role in regulating the expression of subsequent gene products. Expression of the MIE gene is under the control of the powerful and complex major immediate early promoter (MIEP). The MIEP activity is regulated by cellular and viral proteins acting positively or negatively on the expression of the MIE gene, exhibiting strong cell type specificity in expression. Its activity depends on the presence of appropriate transcription factors in the cells, and is influenced by the modulator region upstream to the enhancer-promoter that confers cell type and differentiation state dependence. The regulatory region extends from 500 bp to 1000 bp and contains cellular factor binding sites (93).

2.3. General properties of myeloid DCs

Dendritic cells (DCs) comprise a family of professional antigen-presenting cells which play a crucial role in initiating an immune response. DCs are continuously produced from hematopoietic stem cells within the bone marrow and divide into subsets characterized by their tissue distribution, morphology, surface markers and functions. The morphology of DCs is unusual: they are irregularly shaped cells that display cell processes. Transmission electronmicroscopic visualization has revealed pleiomorphic nuclei, multivesicular bodies and cytoplasmic protuberances (veils) (50). Subpopulations of human DCs include: two subpopulations in the myeloid lineage, Langerhans cells (LCs) and interstitial DCs, and one in the lymphoid (plasmacytoid) lineage. DCs exist in different functional stages: a maturation process from proliferating DC progenitors through nonproliferating DC precursors and immature, antigen-capturing DCs leading to mature, T cell-stimulatory DCs (75).

Human myeloid DC precursors are present in the bone marrow and in the blood. Immature myeloid DCs (iDCs) are observed in the epidermis (LCs), the dermis, the blood, the interstitial spaces of most tissues (interstitial DCs). After maturation they are present in lymphoid tissues and, in low numbers, in the blood. The most common and convenient source for the *in vitro* isolation and generation of human DCs is the peripheral blood, and especially the blood monocytes of myeloid origin, but DCs have also been generated from other tissues, including the bone marrow, cord blood, thymus, tonsils, skin and synovial fluid (40).

DCs do not divide in culture, even after stimulation with the bacterial cell wall component lipopolysaccharide (LPS) or concanavalin A, or *in vivo*, indicating that they are multipotential end cells with the ability to mature. (78). DCs do not carry stable surface markers, since the DC phenotype changes during their lifetime. The combination of several markers allows a description of a DC subpopulation and its stage of activation/maturation. Since several forms of activation exist, the term activation is usually used to refer to any change from the resting state. The changes can affect the ability of DCs to deliver signals to T cells, and include changes in the expression of surface markers, cytokine production, migratory properties, endocytic activity or morphology. This broad definition allows for the possibility that there may be multiple forms of DC activation with different functional consequences. The molecules expressed by human DCs can be divided into: (1) receptors for antigen uptake, such as Toll-like receptors (TLRs), Fc receptors, CD46, CD150 and C-type lectins , such as DC-specific intracellular adhesion molecule-3-grabbing nonintegrin

(DC-SIGN), (2) migration receptors, such as CD44, chemokine receptors 1, 5, 6 and 7, and CD88, (3) adhesion and co-stimulation molecules, such as CD11a, b and c, CD54, CD58, CD80 (B7.1) and CD86 (B7.2), (4) cytokines, such as IL-6, IL-12, IL-15, IL-18, tumor necrosis factor (TNF) and chemokines, (5) signaling molecules, such as CD40, TNF-R and receptors for other cytokines such as granulocyte-monocyte colony-stimulating factor (GM-CSF), IL-1, IL-4, IL-10 and transforming growth factor (TGF)-β, (6) antigen-processing molecules such as MHC class II and CD1a, b and c, (7) other molecules, such as CD83. Human CD83 is a 45-kDa glycoprotein and a member of the Ig superfamily; it is expressed, together with co-stimulatory molecules, such as CD80 and CD86, on functionally mature DCs. CD83 is required for CD4+ T cell generation, regulates T cell proliferation and increases the proportion of CD8+ T cells. (36, 50, 78, 114).

The most unique function of DCs is their ability to prime naive T cells, i.e. to stimulate a primary immune response, while macrophages and other antigen-presenting cells are involved mainly in the expansion of activated T cells. Thus, DCs mostly circulate in the blood in precursor forms, enter the peripheral tissues as immature DCs, where they capture microbial or other antigens by pinocytosis, macropinocytosis, receptor-mediated endocytosis or phagocytosis, then migrate to the lymphoid tissues and, after maturation, present antigenic peptides in context with MHC I or MHC II molecules (cross-presentation) to the circulating T cells. Activated T cells further induce terminal DC maturation. B lymphocytes can recognize native antigens directly, but DCs also influence B cell proliferation, differentiation and isotype switching (29, 41, 91). However, DCs can present not only foreign antigens, but also self-antigens. Two mechanisms exist to prevent the immune system from attacking the self-components: central and peripheral tolerance, both of which are controlled and maintained by DCs (123).

The fate of pathogens inside the DCs is also diverse. Influenza viruses replicate poorly, but express viral proteins in DCs, eliciting protective immune responses (11). Virulent *Salmonella typhimurium* bacterium is killed in the DCs (100) and the growth of *Chlamydia trachomatis* is aberrant (79). Human immundeficiency virus (HIV) can infect productively the immature DCs which serve as an efficient transport vehicle for the virus. HIV prevent DCs to stimulate T cells, but mature DCs do not support the replication of HIV-1 (66). Measles can replicate in DCs eliciting protective immune responses but cause immunsuppression against other pathogens (66). Some pathogens, such as herpes simplex virus, vaccinia virus can replicate in DCs downregulating MHC-I and MHC-II, thus avoiding presentation of microbial antigens to T cells (87). In some cases e.g. HCMV, EBV, influenza,

vaccinia, HSV-1, the neighboring uninfected DCs undergo maturation upon cross-presentation of viral antigens (3, 19, 72). DCs can provide a safe asylum for several viruses, e.g. the DC system hosts latent HCMV and human herpesvirus 8 (45, 101). Some chronic infections, such as HSV, trigger autoimmunity by inducing inflammation and expression of co-stimulatory molecules.

It is of special interest that mononuclear cells, including DCs, exist in the intima of the carotid arteries of children, examined at the age of 8 weeks to 10 years, at sites subjected to major hemodynamic stress and predisposed to the development of atherosclerosis (124). Additionally, inflammatory activity represented by activated T lymphocytes, DCs, macrophages and a strongly increased MHC II expression in the intima has been found in all arterial specimens at areas predisposed to atherosclerosis in individuals aged 15-34 years, with no clinical symptoms of cardiovascular disease, indicating the involvement of DCs and other immune cells in the early pathogenesis of atherosclerosis (82). Immune cells, i.e. macrophages, DCs, T and B lymphocytes and mast cells, are common in the plaques, where T cells are activated by local antigens presented by DCs and macrophages. Since a heterogenous population of T cells with heterogenous T cell receptors is found in the lesions, it is likely that several antigens, including autoantigens, are present, and can be presented by several HLA alleles. The candidate antigens/autoantigens (not excluding others) are *Cpn*, HCMV, oxidized low density lipoprotein, heat shock protein 60 (hsp60) and β2-glycoprotein Ib (48).

2.4. The interaction of DCs with *Chlamydophila pneumoniae*

The interactions of monocytes or monocyte-derived DCs or macrophages with *Cpn* are not well understood. The presence of the organism has been detected in cultured human monocytes or monocyte-derived macrophages for up to 120 h after *in vitro* infection, with no evidence of multiplication and with the gradual diminution of chlamydial antigens (127). On the other hand, it has been reported that infectious particles and chlamydial transcripts may be detected in human monocyte-derived DCs at various intervals, up to 24 days after *in vitro* infection (126). However, no quantification was applied in this study, thus sufficient information about bacterial growth in DCs has not been provided. In addition, the *Cpn* bacterium and recombinant *Cpn* hsp60, but not *Cpn* hsp10, induced human DC maturation, as assessed by immunophenotypic analysis of surface molecules and the production of regulatory cytokines (6, 126). The phenotype of DC subsets that harbor *Cpn* antigens during the maturation process has not been determined.

IFN-γ has been considered to be a product of T cells and natural killer (NK) cells, but, it was recently reported that, with appropriate stimulation, subpopulations of murine DCs can produce IFN-γ (26). Human monocyte-derived DC cultures stimulated by *Salmonella* (96) or *Mycobacterium* (35) produced IFN-γ, but – to my best knowledge - evidence at the single cell level for human DCs as sources of IFN-γ production has only been provided for *Mycobacterium*-stimulated DCs (35).

Cpn DNA and antigens have been reportedly present in a high percentage of the cellular components of atherosclerotic plaques, including DCs, but only rarely in the normal areas of vessel walls (14). *Cpn* co-localizes with DCs in temporal artery biopsies from patients with giant cell arteritis (122).

2.5. The interaction of DCs with HCMV

Several *in vitro* studies have indicated that the ability of HCMV to replicate in monocyte-derived macrophages is dependent on the state of cellular differentiation. The infection of unstimulated monocytes resulted in the failure of productive virus replication; the block in the virus replication was at the level of the transcriptional or posttranscriptional steps of the expression of the MIE gene (67, 109). Differentiation of monocytes by concanavalin A or allogenic stimulation into macrophages resulted in permissive HCMV infection *in vitro*, but interestingly, reactivation of latent HCMV was shown only in allogenically stimulated monocyte-derived macrophages (112).

Although the role of monocytes/macrophages in the pathogenesis of HCMV infection is well established, few data are available concerning HCMV infection in DCs. HCMV DNA was recently detected in a small percentage of DCs and myeloid cell progenitors of healthy seropositive individuals, and it was suggested that these cells harbor latent HCMV (45). Initially, HCMV binds to the heparan sulfate receptor on the DCs, and the receptors on the DCs to which HCMV binds in the second step of adsorption are suggested to be DC-SIGN or its homolog DC-SIGNR, and the HCMV envelope glycoprotein B is the viral ligand of this receptor (46). HCMV strains adapted to endothelial cells productively infect human DCs, but strains adapted to fibroblasts do not (102); however, it is probably not the HCMV adsorption step that is responsible for the differences in the outcome of viral infection.

Endothelial cell-adapted HCMV not only replicates in DCs, but in infected cells the virus interferes with the maturation and antigen-presenting function of DCs, as observed by downregulation of the MHC class I and II molecules, co-stimulatory molecules, and decreased influenza virus-specific cytotoxic T lymphocyte (CTL) activity and T cell

proliferation. However, in the neighboring HCMV antigen-negative cells of the culture, the expressions of co-stimulatory and HLA-I and II molecules were upregulated, indicating that, in the absence of viral infection, bystander DC activation may occur through the binding of viral products or soluble factors (85).

On the other hand, DCs co-cultured with human fibroblast cells infected with a fibroblast-adapted HCMV strain (AD169) were nonpermissive for HCMV infection but they did induce HCMV-pp65 and MIE antigen-specific T cell responses. The HCMV-infected fibroblasts were not apoptotic, suggesting a mechanism other than apoptosis in the initiation of cross-presentation (115). Interestingly, it was also suggested that DC maturation, including changes in the expression of the CD83 molecule, occurs in the presence of an infected-cell-conditioned medium, indicating the presence of stimulatory molecules in the conditioned medium of the infected cells (4). Since cultured human DCs can take up and present soluble antigens (106), it is possible that not stimulatory molecules, but soluble HCMV antigens released by the infected cells and present in the conditioned medium induced the maturation of the DCs. The latter possibility is not likely, however, since no IFN-γ production of T cells following co-culturing with stimulated DCs was observed in this assay. The CD83 expression was somewhat higher on DCs treated with infected-cell-conditioned medium obtained early (24-48 h) than on DCs treated with such medium obtained late (72 h), and the secretion of transforming growth factor β1 was suggested to be responsible for the inhibition of the enhanced expression of CD83 by the late medium (4).

3. Aims

Our research group has investigated the development of AT in relation with infections and inflammation in humans and mouse model (12, 20, 21, 22, 51, 62, 65, 95, 119, 120, 121).

My work has concentrated on the biology and immunology of repeated *Cpn* infections in mice, and the *in vitro* interactions of human DCs with *Cpn* or HCMV infections (Annex 1, 63, 64, Annex 2, Annex 3).

Specifically,

1. Using a mouse model we addressed the question as to what extent *Cpn* replicates and develops bacterial persistence after primary and re-infections.
2. Our aim was to determine the growth characteristics of *Cpn* in human DCs and the cell types that harbor *Cpn* antigens during the DC maturation process. We also planned quantitative the measurement of the mRNA expression of critical *Cpn* genes in the DCs so as to characterize the state of chlamydial infection in these cells. We additionally raised the question of whether IFN-γ is produced in DC cultures in response to *Cpn* stimuli, possibly modulating the production of infectious progeny by DCs, and analyzed the cellular source of IFN-γ in the DC culture.
3. We raised the question whether a recent HCMV isolate (HCMV-Oslo) which has been passaged in MRC-5 cells 10 times, could replicate in DCs, and whether the infection of iDCs with this strain would modulate the differentiation of iDCs as measured by the expression of maturation signals, and functions, such as the ability to stimulate T lymphocytes. We also aimed to study whether a HCMV-infected cell conditioning medium (HCMV conditioning medium) obtained early (24 h) or late (7-9 days) after the infection of MRC-5 cells would similarly modulate the expression of these maturation markers and the functions of DCs, and whether viral particles are necessary for the modulation.

4. Materials and Methods

4.1. Preparation of *Cpn* inoculum

Cpn strain TW-183 was purchased from the American Type Culture Collection, and was propagated in McCoy cells in Modified Eagle Medium (Sigma), supplemented with 10% fetal calf serum, 0.5% glucose, 0.3 mg L-glutamine, 4 mM HEPES and 25 μg/ml gentamycin. Two hours after infection, the medium was supplemented with 1 μg/ml cycloheximide. Infected cells were harvested on day 3 or 4 and were disrupted by two cycles of freeze-thawing and ultrasonication. After centrifugation at 500g for 10 min to remove cell debris, the bacteria were concentrated by high-speed centrifugation at 30.000g for 30 min. Pellets were resuspended in sucrose-phosphate-glutamic acid buffer. The partially purified and concentrated elementary bodies (EBs) were aliquoted and stored at -75 °C until use. A mock stock suspension was prepared from uninfected McCoy cell monolayers processed in the same way as the infected cells. The titer of the infectious EBs was determined by the inoculation of serial dilutions of the EB suspension onto HEp-2 monolayers, and after 48 h the cells were fixed with acetone and stained in an indirect immunofluorescence (IF) assay with a monoclonal antibody to *Cpn* major outer membrane protein (DAKO) and anti-mouse IgG (Fab-specific) FITC-conjugate (Sigma). The number of *Cpn* inclusion-forming units (IFU) was counted under a Leitz UV microscope (22).

4.2. Animal model

Six to 8-week-old female BALB/c mice were inoculated intranasally one, two or three times under mild anaesthesia (pentobarbital sodium) with 7.5 x 10^5 IFUs of *Cpn* in a volume of 30 μl, or with the same volume of a mock preparation (McCoy cell preparation). Mice receiving a single inoculation were anesthetized and sacrificed, and specimens were taken 1 and 2 days and 1, 2, 4, 8, 10, 16, 18, 24, 36 and 49 weeks after the first inoculation. One group of mice received a second inoculation at week 8, and specimens were taken 1 and 2 days and 1, 2, 4 and 8 weeks later. A further group of mice received a third inoculation 16 weeks after the first one, and samples were taken 1 and 2 days and 1, 2, 4, 8, 20, 28 and 33 weeks later. Three to seven *Cpn*-inoculated mice were sacrificed at one time point, except at weeks 36 and 49, when only one or two mice, respectively, were sacrificed from the group inoculated only once. Two of the mock-inoculated mice were sacrificed at one time point, except at weeks 20 and 33, when only one mouse was sacrificed from the group that received three inoculations.

Heparinised blood samples were collected by heart puncture for PCR analysis. The lungs were removed and homogenized mechanically in 2 ml of sucrose-phosphate-glutamic acid buffer for PCR, cultivation of bacteria and cytokine measurements.

4.3. Culturing of *Cpn* from the lungs

Lung homogenates from individual mice were centrifuged (10 min, 400g), serial dilutions of the supernatants were inoculated onto McCoy cell monolayer and the titer (IFU/ml) of *Cpn* was determined as described for the preparation of the *Cpn* inoculum.

4.4. Detection of *Cpn* DNA in the lungs and blood

DNA was extracted from the whole blood, lung and spleen of the mice with a Wizard Genomic DNA purification kit (Promega).

In nested PCR (nPCR) *Cpn ompA* gene specific primer pairs were used. The sequences of primer pairs were as follows: *Cpn MOMP* outer: F: 5'-TTACAAGCCTTGCCTGTAGG-3', R: 5'-GCGATCCCAAATGTTTAAGGC-3'; *Cpn MOMP* inner: F: 5'-TTATTAATTGATGGTACAATA-3', R: 5'-ATCTACGGCAGTAGTATAGT-3'. The nPCR conditions with outer primer pairs were as follows: after initial denaturation 3 min at 95 °C, 15 amplification cycles for 30 s at 95 °C, 30 s at 50 °C and 60 s at 72 °C followed by 20 amplification cycles for 30 s at 95 °C, 30 s at 55 °C and 60 s at 72 °C. The nPCR conditions with inner primer pair was the following: initial denaturation 3 min at 95 °C, followed by 35 amplification cycles for 30 s at 95 °C, 30 s at 55 °C and 60 s at 72 °C. The final extension of both primer pairs was 5 min at 72 °C. DNA from the blood of individual mice was subjected to 4 replicate PCR runs. The DNA from the lungs and spleen was tested in a three and a single replicate PCR reactions, respectively. The presence of PCR inhibitors in the samples was excluded by the amplification of mouse β-actin from each sample in a simple PCR. False-positive reactions were highly improbable, since all of the PCR-negative controls (every fifth reaction) and samples from mock-inoculated mice were negative. Samples were considered positive when at least 1 of the nPCR replicates was positive.

Real-time PCR was performed as described in Section 4.12. The limit of detectability was 6.7×10^2 copy numbers/ml. For each sample analyzed, the relative number of *Cpn* genome equivalents was established. The lungs obtained from a mock-infected mouse and spiked *in vitro* with *Cpn* at one inoculation dose showed a copy number of 1.2×10^9/ml.

4.5. Preparation of HCMV conditioning media

The HCMV strain Oslo was isolated in human fibroblast cells at the Institute of Medical Microbiology, Rikshospitalet University Hospital, Oslo, Norway (56), and provided by Dr. Miklos Degre. For the preparation of HCMV conditioning medium, a human fibroblast culture, MRC-5 (Coriel, USA) were infected at a multiplicity of infection (MOI) of 0.1. After adsorption for 1 h, the monolayers were washed and fresh medium was added. The medium was collected from the infected cultures on day 1 (early HCMV conditioning medium) and fresh medium was added to the cultures. To prepare the late HCMV conditioning medium, the culture medium was removed on day 7, fresh medium was added and collected on day 9 (late HCMV conditioning medium). The media were clarified from the cell debris by centrifugation (800g, 10 min), aliquoted and stored at -70 °C. Early and late mock conditioning media were obtained from uninfected MRC-5 cells in a similar way. Virus particle-poor conditioning media were prepared by ultracentrifugation (1 h, 100,000 g) following low-speed clarification of the conditioning media. The infectious virus titer was determined by plaque titration. The titer of the late conditioning medium before ultracentrifugation was 1-2 x 10^7 plaque forming units/ml. No infectious particles were detected in the ultracentrifuged, undiluted supernatants of the late conditioning medium by plaque formation during the 14-day observation period of the inoculated MRC-5 cells, using 200 µl/well inoculum in 5 parallels, indicating less than 1 infectious particle/ml in these preparations. In addition, labeling of MRC-5 cells at 48 h after exposure to the ultracentrifuged undiluted late conditioning medium with a monoclonal antibody specific for HCMV-MIE antigen (Imagen CMV; Dako) in an IF assay, revealed no cells expressing the HCMV-MIE antigen.

4.6. Preparation of DC cultures

PBMCs were isolated by Ficoll-Paque PLUS (Amersham) density gradient centrifugation of buffy coats obtained from healthy blood donors. The HCMV or *Cpn* serostatus of the donors was determined by an IgG ELISA (DADE Behring, or NovaTec Immunodiagnostica, respectively). DCs were generated from blood monocytes by the adherence method (106, 115). The nonadherent fraction of PBMCs was collected and cryopreserved until use. The adherent cells were maintained in RPMI 1640 (Sigma) containing 10% fetal bovine serum (Sigma). After 24 h, the loosely adherent and detached cells were collected and seeded in culture medium supplemented with 800 U/ml recombinant

human GM-CSF and 1000 U/ml recombinant human IL-4 (R&D Systems Inc.); these cytokines were added to the cells every 2-3 days. At day 7 the purity of the iDCs was about 90%, as determined by an IF test with the lineage cocktail and flow cytofluorometry.

4.7. Detection of maturation markers in DCs

To assess the expression of surface molecules on the DCs, 1-2 x 10^5 DCs were labeled with antibodies as recommended by the manufacturers. The following antibodies and fluorescent reagents were used: CD14-PE, HLA-DR-PerCP, CD11c-APC (BD Pharmingen), CD1a-PE (DAKO), CD86-PE and CD83-APC (Caltag). To exclude the presence of T and B lymphocytes, NK cells, monocytes and macrophages in the population gated for DCs, a lineage cocktail containing FITC-labeled antibodies specific for CD3, CD19, CD20, CD56, CD14 and CD16 molecules (BD Pharmingen) was included in the antibody mixture. Maturation markers on the activated DCs were determined by three- or four-color IF cell-surface staining after gating forward and side scatter by flow cytofluorometry, or by staining with the lineage cocktail. For cytofluorometry, 10.000 events were acquired by live gating on a FACS Calibur (Becton Dickinson) and analyzed with CELLQuest Pro software. The percentages of positive cells and the fluorescence intensities of the investigated markers (mean channel values) were compared.

4.8. Infection of DCs with *Cpn*

On day 7 of the DCs preparation, the nonadherent and loosely adherent immature DCs (iDCs) were collected, counted, centrifuged, resuspended and seeded in 24-well plates at a density of 6-7 x 10^5 cells/well. The iDCs were infected with *Cpn* at an MOI of 2 IFU/cell, unless otherwise stated in the Results and Discussion section, or with the same volume of mock stock (control cultures), then centrifuged at 600g for 45 min at 25 ^oC. After centrifugation, the medium was gently changed to RPMI-1640 containing 4 mg/ml glucose and 25 μg/ml gentamicin. Every 4^{th} day, half of the supernatant was replaced with fresh medium. DC surface markers were tested on days 1, 7 and 10 after initiation of the cultures. The viability of cells with DC morphology was determined by staining with propidium iodide (10 μg/ml) immediately before flow cytofluorometry analysis. A MOI of 2 was used in most of the experiments, as a compromise between a higher rate of infection, but impaired viability of the DCs at higher MOI values. At an MOI of 2, the viability of the *Cpn*-infected DCs gradually decreased from 72% (4 hours) to 20% (6 days), while that of the mock

infected DCs decreased from 81% (4 hours) to 48% (6 days) after infection (donor 141, with similar results using DCs of 6 additional donors).

4.9. Detection of *Cpn* antigens in DCs

The presence of *Cpn* antigens in the DCs was determined by direct surface staining of cells with antibodies to CD83, CD86 and HLA-DR, and then, after fixation and blockade of the nonspecific binding with rabbit serum and permeabilization with 0.1% saponin, the cells were stained with *Cpn*-positive human serum and FITC-labeled anti-human conjugate (DAKO). The results were read by flow cytofluorometry.

4.10. Determination of *Cpn* infectivity in DCs

DCs were harvested at different time points, and serial dilutions of the cells lysed into their culture medium (three freeze-thaw cycles and sonication) were inoculated onto HEp-2 cell monolayers and the titer (IFU/ml) of *Cpn* was determined as described for the preparation of *Cpn* inoculum.

4.11. Stimulation of CD4+ and CD8+ lymphocytes by DCs – two experimental approaches

We used two experimental approches to reveal the ability of DCs to stimulate T lymphocytes.

The lymphoproliferative response was assessed by determining the frequency of CD4+ and CD8+ cells incorporating bromodeoxyuridine (BrdU) by using a BrdU labeling kit. Briefly, the iDCs were treated with HCMV or with *Cpn* or with mock preparations for 3 days, then thoroughly washed with phosphate buffer solution (activated DCs). One million autologuous lymphocytes were incubated for 5 days with 5 x 10^4 activated DCs. BrdU was added 18 h before harvesting. The incorporation of BrdU was measured by using anti-CD4-PerCP, anti-CD8-PE and anti-BrdU-FITC antibodies (all from BD Pharmingen) in three-color IF and flow cytofluorometry according to the manufacturer's protocol. A subset of lymphocytes was directly stimulated with HCMV or *Cpn* or mock preparation without the presence of DCs, and the percentages of CD4+ or CD8+ cells were subtracted from the data obtained following the coculturing of the lymphocytes with activated DCs.

The IFN-γ production of CD4+ and CD8+ lymphocytes was determined with an IFN-γ secretion assay as suggested by the manufacturer (MACS Secretion Assays for Human Cells, Miltenyi Biotec). Briefly, 10^6 autologuous lymphocytes were cocultured with 5 x 10^4

activated DCs for 6 h then the percentage of IFN-γ producing CD4+ and CD8+ cells were determined according to manufacturer's instruction using anti-IFN-γ-FITC and anti-CD4-PerCP or anti-CD8-PE antibodies and the three-color IF; the assay was read by cytofluorometry. Similarly to the BrdU-incorporation experiments, a subset of lymphocytes were directly stimulated with the HCMV or mock conditioning medium without the presence of DCs, and the percentages of CD4+ or CD8+ lymphocytes producing IFN-γ were subtracted from the data obtained following the coculturing of lymphocytes with activated DCs.

4.12. Expression of mRNA of chlamydial genes in DC cultures as determined by real-time PCR

Infected cells were collected at different time points, washed and frozen at –75 °C until isolation with a High Pure RNA Isolation Kit (Roche), followed by DNase treatment with deoxyribonuclease I (Sigma), according to the manufacturers' instructions. The absence of bacterial DNA was confirmed by real-time PCR specific for the chlamydial *16S rRNA* gene. Reverse transcription reactions were performed with the Enhanced Avian First Strand Synthesis Kit (Sigma). The expression profiles of some of the chlamydial genes (*16S rRNA*, *omcB*, *groEL-1* and *ftsK*) at different time points after infection were investigated. In order to measure the cDNA copy number of these genes, nested primer pairs for the *16S rRNA*, *groEL-1* and *omcB* were designed by using the DNA Lasergene program (DNASTAR Inc), BLAST search and synthesized (Integrated DNA Technologies Inc). The inner primers were used for the real-time quantitative PCR: *16S rRNA*: F: 5'-AAA GCT AGC CCC AGT TCG GAT TGT-3', R: 5'-CAT GAT GTG ACG GGC GGT GTG-3'; *groEL-1*: F: 5'-AAA GCC GTA AAA GTT GTT GTT GAT-3', R: 5'-ACG CAT TCT TGA GTT TCT GAA TT-3'; *omcB*: F: 5'-GTG ATG GGA AAT TAG TCT GG-3', R: 5'-ATC CTG TGT TCA CTA CTT CG-3'. The *ftsK*-specific inner primers were described earlier (23).

Amplicon standards were generated by amplifying genomic *Cpn* DNA with the outer primers: *16S rRNA*: F: 5'-GCA ACG AGC GCA ACC CTT ATC-3', R: 5'-CAT CCC AGT CAT CAG CCT CAC CTT-3'; *groEL-1*: F: 5'-CAG GCG ACG GAA CTA CAA CAG C-3', R: 5'-TGA TTA AAA GAG GGC GTC CAG ATT-3'; *omcB*: F: 5'-TAT GAC AGC GAA GAA GGT TAG A-3', R: 5'-TAC AGT TAC GTT ACG GGC AAT AG-3'; and *ftsK* (23).

Amplicons were purified with the PCR Clean-up Kit (V-gene Biotechnology Ltd.), DNA concentrations were measured with a spectrophotometer (Perkin Elmer Lambda UV/VIS), and the copy numbers were calculated and standard curves generated from serial

dilutions of the amplicons. The LightCycler real-time PCR system (Roche) and the LightCycler FastStart DNA Master SYBR Green I kit were used according to the manufacturer's instructions. The minimal template concentration which consequently gave positive PCR results was 2-20 copies/μl of cDNAs. The conditions of the real-time PCR were as follows: initial denaturation and enzyme activation for 10 min at 95 °C, followed by 50 amplification cycles for 3 s at 95 °C, 8 s at 57-63 °C (depending on the primer pairs) and 12 s at 72 °C. Melting curve analysis and gel electrophoresis were used to determine the specificity of the PCR products. The mean cDNA copy number obtained for each gene was divided by the corresponding mean *16S rRNA* value to standardize for the number of metabolically active chlamydial bodies present in each sample (relative copy number) as described earlier (97).

4.13. IFN-γ production in DC cultures

To assess the production of IFN-γ protein in infected DCs, two experimental approaches were used. One was to measure the cytokine level in the supernatants of *Cpn*-infected DC cultures by ELISA (OptEIA; BD Pharmingen) according to the manufacturer's instructions. The sensitivity of the IFN-γ measurement was 9.4 pg/ml. IFN-γ was neutralized by supplementing the culture medium with 20 μl/ml of an anti-human IFN-γ monoclonal antibody (Serotec) for 5 days. The neutralization was confirmed by the ELISA detection of <9.4 pg/ml IFN-γ in the antibody-treated cultures.

In the other approach, the presence of IFN-γ inside the DCs was determined by IF and flow cytofluorometry. Briefly, GolgiPlug (BD Pharmingen, 10 μg/10^6 cells) was added 6 h before harvesting DCs to prevent the release of cytokine. After collecting and washing, the cells were stained with the lineage cocktail and antibodies to the CD11c and HLA-DR surface molecules, and the cells were then fixed with paraformaldehyde, permeabilized with 0.5% saponin and stained with PE-labeled mouse anti-human IFN-γ antibody (BD Pharmingen).

4.14. Treatment of DCs with HCMV preparations

On day 7 of the DCs preparation, nonadherent cells (iDCs) were collected, counted, centrifuged, resuspended in HCMV or mock conditioning medium, without or with TNF-α (50 ng/ml), or in the ultracentrifuged supernatant of the HCMV or mock conditioning media, and seeded in 24-well plates at a density of 7-8 x 10^5 cells/well. Next day a half volume of fresh medium was added to the cells. Surface markers were tested on days 1, 7 and 10 after

initiation of the DC cultures (see section 4.7), and the functions of DCs were tested on day 10 (see section 4.11).

4.15. Detection of HCMV antigens in DCs

The presence of HCMV-MIE antigens in the DCs was examined by IF microscopy, using cytospin preparations of infected DCs that were fixed in acetone and stained with the FITC-labelled anti-MIE monoclonal antibody (Imagen CMV, DAKO).

5. Results and Discussion

5.1. *Cpn* growth in mice following repeated infection with *Cpn*

5.1.1. Detection of live bacteria in the lung

In order to establish a mouse model for chronic *Cpn*-infection, BALB/c mice were inoculated intranasally with *Cpn* or with the same volume of the mock preparation and the primary infection was followed by two re-inoculations at 8-week intervals. The mice showed signs of sickness, ruffled fur and a decreased body weight from day 3 to day 14 after the primary infection. These symptoms were less pronounced and shorter-lasting after the second infection, and were absent after the third one. All of the mice recovered after the infections. Viable bacteria were cultured from the lungs of mice on days 2, 7 and 14 after the primary inoculation, resulting in the peak titer on day 7 post-infection [geometric mean (GM) 6.9 x 10^4 IFU/lung] (Fig. 1). By week 4 and at later time points after inoculations, no viable *Cpn* could be recovered (not shown). The numbers of bacteria were similar on day 2 after both re-infections to those on day 2 after the primary inoculation, but as a sign of significant protection against the infection, culturable *Cpn* was cleared from the lungs by day 7 after the re-inoculations (Fig.1).

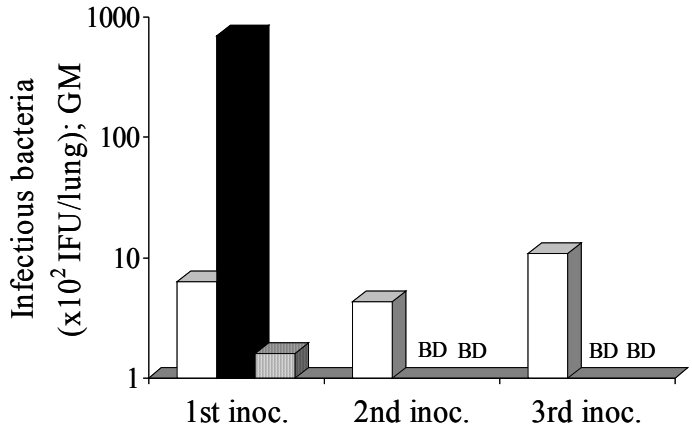

Fig. 1. Recovery of culturable *Cpn* from the lungs of mice after 1, 2 or 3 *Cpn* inoculations. Lung homogenates were inoculated onto McCoy cell monolayers and chlamydial inclusions were detected by iIF using MOMP-specific monoclonal and FITC-labelled secondary antibodies. Bars denote GM *Cpn* titers in the lung homogenates of 3-5 mice. BD: below detectability. Day 2: □; Day 7: ■; Day 14: ▓.

5.1.2. Detection and quantification of bacterial DNA in the lung, blood and spleen

After the primary infection, *Cpn* DNA was amplified by *ompA*-specific nPCR from the lungs of all mice from day 2 to 4 weeks, and the number of PCR-positive mice started to decline at 8 weeks. After the second and third challenge inoculations, the frequency of PCR-positivity of the lungs began to decrease at 2 weeks (Table 1). To confirm the presence and to quantify the *Cpn* genomes in the samples, the copy number of the chlamydial *groEL-1* gene that is present in one copy in a single operon in the *Cpn* genome, was determined by real-time PCR in samples positive by nPCR. On day 7, the number of genome equivalents was 100-fold higher after the primary inoculation and about 100-fold lower after the second and third inoculations than on day 1. However, bacterial genomes were still present in a number of 5×10^2 - 7.8×10^3 in 14 of the 25 mouse lungs tested 4-8 weeks after the infections. *Cpn* DNA, tested in 4 replicates by nPCR, appeared in the blood as early as 1-2 days after the primary and the repeated infections. All of the blood samples were nPCR-positive 1 week after the primary infection, and 2 days after the second infection; only 1 of 3 mice was nPCR-positive on days 1 and 2 after the third inoculation. At 2 weeks after the primary infection, 2 of 5 mice were nPCR-positive, as was 1 of 5 mice after the re-infections. In the blood the maximum copy numbers were similar after all three inoculations ($1.0 \times 10^4 – 4 \times 10^4$/ml) (Table 1).

DNA was cleared from the lungs of all mice by week 10 post-infection; however, 1 of 5 mice was PCR-positive in the lung at 24 weeks after the primary infection, and 2 of the 21 tested lungs were positive at 20 and 33 weeks after the third inoculation, at a copy number of 2×10^3-3×10^3/ml suspension (not shown). In some mice left without a re-challenge, *Cpn* DNA was still detectable in the blood at later time points; 3 of 18 tested mice carried *Cpn* DNA in their blood at 10, 18, and 49 weeks post-infection, at a copy number of 1×10^3 - $5.5\ 10^4$/ml (not shown), demonstrating that a small proportion of the animals carry the bacterium in the blood for a long time. *Cpn* DNA was amplified intermittently from the spleen, only in the acute phase of infection (not shown).

Time after post infection	Number of inoculations					
	1		**2**		**3**	
	Lung	Blood	Lung	Blood	Lung	blood
1d	(2+/3) 3.8×10^7 $\pm 2.1 \times 10^7$	(1+/3) $< 6.7 \times 10^2$	(3+/3) 5.4×10^6 $\pm 3.2 \times 10^6$	(0+/3)	(3+/3) 5.6×10^6 $\pm 4.0 \times 10^6$	(1+/3) 7.0×10^3
2d	(3+/3) 1.7×10^9 $\pm 5.6 \times 10^8$	(2+/3) 4.3×10^3 $\pm 2.7 \times 10^3$	(3+/3) 6.3×10^7 $\pm 5.5 \times 10^7$	(3+/3) 1.0×10^4 $\pm 8.2 \times 10^3$	(3+/3) 1.6×10^7 $\pm 1.2 \times 10^7$	(1+/3) 1.0×10^4
1w	(3+/3) 3.8×10^9 $\pm 2.0 \times 10^9$	(3+/3) $< 6.7 \times 10^2$	(3+/3) 5.6×10^4 $\pm 5.4 \times 10^4$	(0+/3)	(3+/3) 7.6×10^4 $\pm 7.1 \times 10^4$	(0+/3)
2w	(5+/5) 1.5×10^7 $\pm 9.2 \times 10^6$	(2+/5) 2.8×10^4 $\pm 2.4 \times 10^4$	(4+/5) 1.8×10^3 $\pm 1.0 \times 10^3$	(1+/5) $< 6.7 \times 10^2$	(3/5) 1.2×10^3 $\pm 1.0 \times 10^3$	(1+/5) 4.0×10^4
4w	(5+/5) 3.8×10^3 $\pm 1.2 \times 10^3$	(0+/5)	(1+/5) 5.0×10^2	(0+/5)	(3+/5) 2.2×10^3 $\pm 3.8 \times 10^3$	(0+/5)
8w	(4+/5) 7.8×10^3 $\pm 8.9 \times 10^3$	(0+/5)	(1+/5) 1.0×10^3	(0+/5)	N.D.	(0+/5)

Table 1. Number of lung and blood samples positive by nPCR and number of genome copies/ml in the samples tested positive by nPCR after 1, 2 or 3 inoculations of mice. The number of lung and blood samples tested positive by *omp A*-specific nPCR/the total number of tested samples are shown in parenthesis. The number of genome equivalents was determined by *groEL-1*-specific real-time PCR; the numbers indicate the mean copy numbers ± SD of *Cpn* chromosomes in 1 ml of lung or blood samples of the nPCR positive mice. N.D. = not done.

In our mouse model, the 2-month periods between the 3 inoculations allowed the mice to recover fully from the clinical signs of the disease; they therefore simulate the possible re-infection frequency in people, since epidemics generally occur every 4-6 years (90). The relatively low inoculation dose of *Cpn* might be closer to those in natural human infections than the much higher doses used for inoculation of mice in some other studies (13,

25, 129). In our model, similarly as reported earlier (60, 94), viable bacteria were eliminated from the lungs more rapidly after re-infection than after the first infection. However, the partial protection remained at the same level after the second re-challenge, thus the immune response boosted by the first re-infection was not effective enough to prevent productive infection in the lungs. Most *Cpn* infections in adults are re-infections and re-infected patients have a milder disease than those with a primary infection (117), although more severe re-infections are observed in older patients (34). Our results showed that the clearance of *Cpn* DNA from the lungs was far less complete than that of viable bacteria, indicating a tendency of *Cpn* to persist in the lung long after infection. Long-term carriage was observed in a few mice. The higher number of genome equivalents than IFUs in the lung suggests the presence of non-infectious bacterial particles in the inoculum, and perhaps also their production in the lungs.

The effects of *Cpn* infection are not limited to the respiratory tract. Infected macrophages in the inflammatory infiltrate of the lung and the dissemination of *Cpn* into the spleen and heart after intranasal inoculation have been reported (130). *Cpn* DNA in the PBMCs was detected in mice after a single infection but not after repeated inoculations (83). In our study the rate of nPCR positivity and the number of genome equivalents declined faster after the second and third challenges than after the first inoculation. However, we were also able to demonstrate *Cpn* DNA in the blood intermittently at later time points. As far as we are aware, the quantification of DNA in the blood after primary and re-infections and during persistency in animal models has not been documented earlier.

Variable proportions of apparently healthy blood donors have been reported to carry *Cpn* DNA in their blood (111). In another study a standard curve of the dilutions of EBs indicated that the range of bacterial levels in the human blood samples was 3-179 bacteria/ml blood, though the number of non-infectious particles was not included in the calculation, thus the precise number of genome equivalents in humans is not known (58).

In addition to the determination of the *Cpn* genome equivalents during acute and chronic infections, the level of histamine, IFN-γ, IL-6, *Cpn* antibodies including chlamydial heat-shock protein antibodies, as well as the number of IFN-γ-producing splenocytes were monitored in the infected and the reinfected mice (Annex 1). However, for the sake of a concise summary of the relevant data, these results are not included in the Thesis.

5.2. Interactions of DCs with *Cpn*

5.2.1. Infectious EBs in the infected DCs

To determine the production of infectious EBs in DCs, iDCs were infected with *Cpn* at an MOI of 0.5-3 and examined for the number of infectious particles by inoculation of serial dilutions of the DC lysates onto HEp-2 cells. As controls, susceptible HEp-2 cells were also infected at an MOI of 1, and the production of EBs was similarly determined. Figure 2 demonstrates that infectious bacterial particles were present in all of the DC lysates up to 24-48 h after inoculation, but the number of these particles decreased after infection, with no detectability (3 donors), or with the presence of a low level (10-50 IFU/ml) of infectious particles 72 and 96 h after infection (2 donors), regardless of the MOI used for the infection. These results indicate the survival or low-level growth of infectious bacteria in some of the DCs. Figure 2 also reveals that the number of IFUs produced by the control infected HEp-2 cells slightly decreased until 24 h, but was increased at 48-72 h after infection, indicating the production of infectious EBs in susceptible cells.

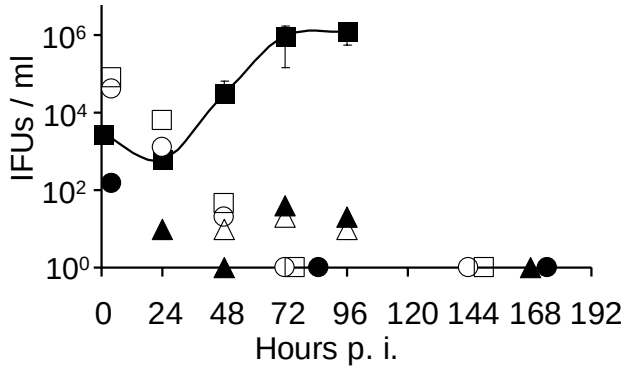

Fig. 2. Infectious EBs in DCs. Immature DCs obtained from 5 different donors were infected with *Cpn* and the production of infectious EBs was determined on HEp-2 cells. A decreasing number of IFUs was observed between 4 and 48 h in all of the donors, but no detectable infectious bacteria at 72 h or later in DC lysates of 3 donors, or a low IFU/ml in the DC lysates of 2 donors at 72 and 96 h after infection. HEp-2 cells infected with *Cpn* produced an increasing number of IFUs, reaching the maximum titer of 1 x 10^6/ml at 72 h after infection. ■ HEp-2 cells; ● DCs, donor 110; ○ DCs, donor 121; □, ▲, Δ DCs, 3 additional donors.

We did not observe an exponential production of infectious EBs by the infected DC cultures. As far as we know, only one research group has investigated the production of infectious progeny in human DCs, obtained from 3 donors. It was found that infectious progeny was recovered at various intervals, up to 24 days after infection, with a great variability in the number of EBs (0-1000 IFU/ml) at different time points after infection, but

there was no exponential growth of the bacteria (126). Our results also indicate that there is no exponential growth of bacteria in DCs, and point to the persistence of bacteria, or occasional replication, in a low number of DCs.

5.2.2. Maturation and functional activation of DCs treated with *Cpn* preparations

To explore whether *Cpn* infection of iDCs would stimulate the expression of certain surface molecules, such as the co-stimulatory molecule CD86, the maturation markers CD83, CD11c and HLA-DR, iDCs were infected with *Cpn* or mock preparation on day 7 and the percentage of live DCs expressing the surface molecules was determined on day 10. Figure 3A shows the percentage of DCs expressing these molecules to be 2.2% (mock-treated) and 68% (*Cpn*-infected) for CD83, 11.8% (mock-treated) and 75.2% (*Cpn*-infected) for CD86, fluorescence intensity 620 (mock-treated) and 957 (*Cpn*-infected) for CD11c, and 2795 (mock) and 4353 (*Cpn*) for HLA-DR. Higher than 95% of the DCs were regularly positive for the expression of HLA-DR and CD11c, regardless of the stimulation with *Cpn* (not shown). These results were confirmed by experiments with DCs obtained from 6 additional donors (4 donors were seropositive and 2 donors were seronegative for *Cpn*) with increased expressions of the examined molecules, although the rates of stimulation exhibited some individual differences. We wanted to know the distribution of the cells harboring *Cpn* antigens in the CD83-positive, CD86-positive and HLA-DR-positive populations. The results showed that *Cpn*-positive cells were seen among the CD83-positive and CD83-negative cells, among the CD86-positive and CD86-negative cells, and among the HLA-DR-positive cells and among cells expressing all three surface markers, indicating that bacterial antigens are present in the DCs irrespective of the stage of their maturation (Fig. 3B). It should be noted that all of the CD83+ cells were positive for CD86 and HLA-DR expression (not shown). DCs treated with the *Cpn* preparation became functionally mature, *i.e*. they were able to stimulate T lymphocytes. DCs obtained from a seropositive donor and treated with the *Cpn* preparation stimulated higher percentages of CD4+ and CD8+ cells to incorporate BrdU (9.8% and 5.6%, respectively) than did DCs treated with the mock preparation (3.2% and 2.6%, respectively). Lymphocytes of a seronegative donor were unable to elicit proliferative responses after incubation with *Cpn*-treated DCs for 5 days. Similar results were obtained when DCs from 5 different donors were used in additional independent experiments.

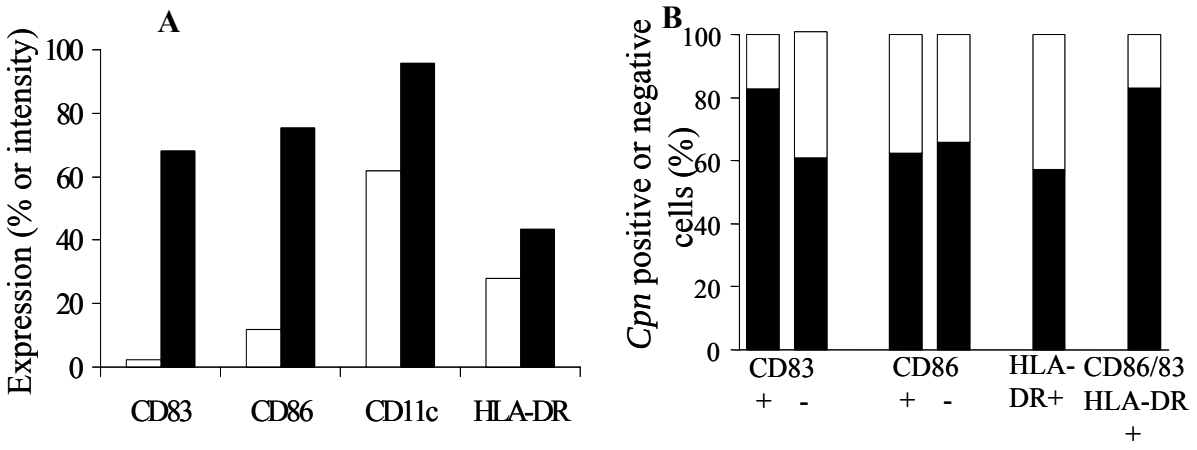

Fig. 3. Expression of CD83, CD86 and HLA-DR molecules on DCs infected with *Cpn* or mock preparation, and the distribution of *Cpn*-positive cells. Panel A. Immature DCs were infected with *Cpn* on day 7 and tested for the expressions of CD86, CD83, CD11c and HLA-DR by four-color IF and flow cytofluorometry on day 10. The cells negative for surface markers of T and B lymphocytes, NK cells and monocytes/macrophages (lineage cocktail) were read for the expression of CD86, CD83 (%) and CD11c, HLA-DR (intensity, reduced 10 or 100 fold, respectively). ■ *Cpn*-infected cells; □ mock-infected cells.
Panel B. Distribution of *Cpn*-positive and negative cells in CD83, CD86 and HLA-DR positive or negative DC populations (%). A low percentage of the mock-infected DCs exhibited a nonspecific staining of *Cpn* antigens; this percentage was subtracted from that for the *Cpn*-infected cultures. ■ % of cells harboring *Cpn* antigens; □ % of cells with no *Cpn* antigens.

Our results revealed that the infection of DCs with *Cpn* modulated DC maturation, as assayed by the increased expression of costimulatory molecules, and functions such as the stimulation of autologous lymphocytes. We identified cells in the DC population harboring *Cpn* antigens as CD83-positive, CD83-negative, CD86-positive, CD86-negative and HLA-DR-positive, or DCs expressing all of these molecules during the maturation process. These results indicate that *Cpn* infection is not exclusive for any of the investigated subpopulations of DCs. Interestingly, treatment of the DCs with heat-inactivated *Cpn* (60 ^{o}C, 45 min) induced a strong increase in the expression of costimulatory molecules and an elevated stimulation of autologous lymphocytes by the DCs (not shown). However, we have not explored whether the DC maturation statuses induced in response to live or heat-inactivated organisms are identical or distinct. The mechanism of DC maturation by pathogens such as *Cpn* is not clear. A recombinant chlamydial hsp60 induced a strong DC maturation, as assessed by the expression of co-stimulatory molecules, and a preferential Th1 polarization of immune response due to the expression of IL-12 and IL-23. It has also been shown that an ultrapure recombinant *Cpn* hsp60 signals through TLR-4 in monocytes and epithelial cells, supporting the direct stimulation of DCs (6, 77). Further, anti-CD3-activated

T cells produced several soluble factors, including CD40-ligand, TNF-α, and IFN-γ, that markedly increased the expression of co-stimulatory molecules on DCs, indicating that these factors can also be responsible for the maturation of DCs (59). Thus, either direct stimulation by *Cpn* or *Cpn* hsp60 through TLRs, or an indirect effect by the products of the cells present in the *in vitro* cultures, or both mechanisms, are involved in the maturation process by *Cpn* infection.

5.2.3. cDNA copy number of selected *Cpn* genes in DCs

The presence of chlamydial transcripts has been determined in *in vitro* infected DCs (126). However, EBs frequently contain mRNA from previous developmental cycles (10, 108), thus without quantitative analysis evidence for the primary bacterial mRNA expression in *Cpn*-infected DCs cannot be provided. In our study DC cultures were infected with *Cpn* and the mRNA expressions of *16S rRNA*, *groEL-1*, *omcB* and *ftsK* were investigated quantitatively by real-time PCR in cells harvested at different times after infection. The *groEL-1* gene, coding for the 60 kD bacterial heat shock protein, is transcribed from early time points in the developmental cycle, while *omcB*, which encodes a cysteine-rich outer envelope structural protein, is expressed late in infection (10, 108). The *ftsK* gene is related to bacterial division and has previously been shown to be down-regulated in certain *Cpn* persistence models (23, 110). *16S rRNA* transcripts are often used as a measure of the growth rate and/or the number of metabolically active organisms, and are considered a useful means for the normalization of transcriptional data (97). Figure 4A shows that the copy number of the *16S rRNA* transcripts in the DC cultures was detected at a steady-state level, or with a decrease of less than 10-fold, during the observation period of 7 days. The expression of the *16S rRNA* transcripts in the DCs (Fig. 4A) was confirmed by demonstrating a roughly 10-fold decrease in the copy number of *16S rRNA* in all 3 concentration of a chlamydial suspension incubated at 37 ^{o}C for 7 days without the presence of any eukaryotic cells (Fig. 4B). The relative copy numbers of the *groEL-1*, *omcB* and *ftsK* genes were under the level of detectability in the chlamydial suspension at 24 h of incubation without eukaryotic cells (not shown).

The relative copy numbers of the *groEL-1* and *omcB* genes in DCs increased within 72 h, and then decreased, but remained at the level of detectability throughout the observation period, as illustrated for 2 donors (Fig. 4, panels C and D). The data on donors 110 and 121 are included in Fig. 2 and 4 A, C and D, demonstrating no production of infectious EBs, but the mRNA expression of the investigated genes in the DCs of the same

donors. The mRNA expression of the *ftsK* gene was at the level of detectability during the observation period of 7 days in 4, and below the level of detectability in 3 of the donors (not shown). Since the growth of *Cpn* in lymphocytes obtained from some, but not all investigated donors was reported earlier (49), to discover whether the presence of the bacterial transcripts in the DC cultures is due to the contaminating lymphocytes expressing chlamydial mRNA, the lymphocyte populations of 4 donors separated and cryopreserved at 2 h after initiation of the DC cultures were reconstituted on day 6, infected with *Cpn* concomitantly with the DC population and assayed for the mRNA expression of the *16S rRNA*, *groEL-1* and *omcB* genes. The copy number of *16S rRNA*, and the relative copy numbers of the *groEL-1* and *omcB* transcripts in the lymphocytes were always about 10 times lower than in the same number of cells in the DC cultures during the observation period (not shown). In our control *Cpn*-infected HEp-2 cells, the expressions of the investigated genes were readily detectable. The copy number of the 16 S rRNA transcripts had increased 10-fold by 48 h after infection (Fig. 4A). The relative copy numbers of the *groEL-1*, *omcB* and *ftsK* mRNA increased after infection; it increased for the *groEL-1* gene from 8 h up to 72 h, for the *omcB* gene from 24 h and peaked at 48 h, and for the *ftsK* gene from 8 h to 24 h (not shown), as described previously (55, 97).

Our results show that DCs infected with *Cpn* express mRNAs from several critical bacterial genes such as the *16S rRNA*, the *groEL-1* and the *omcB* genes (linked to bacterial growth), with some individual differences in the donors, but with only a weak expression of the *ftsK* gene (involved in bacterial division), similarly to other *Cpn* persistence models (23, 110). However, *ftsK* expression was not decreased in a continuous persistence model of *Cpn* in HEp-2 cells (55), indicating that different mechanisms can be responsible for persistence. The expression of some, but a restricted expression of other bacterial genes in DCs accords with the not exponential, but occasional production of infectious EBs. A restricted bacterial mRNA expression and an occasional production of infectious EBs, as demonstrated in our study, indicate that the DCs play a major role in the development of chronic *Cpn* infections by providing a continuous antigenic stimulus to inflammatory cells co-localized with the DCs. The DCs may also serve as a source for the production of live bacteria, thereby contributing to the dissemination of the infection.

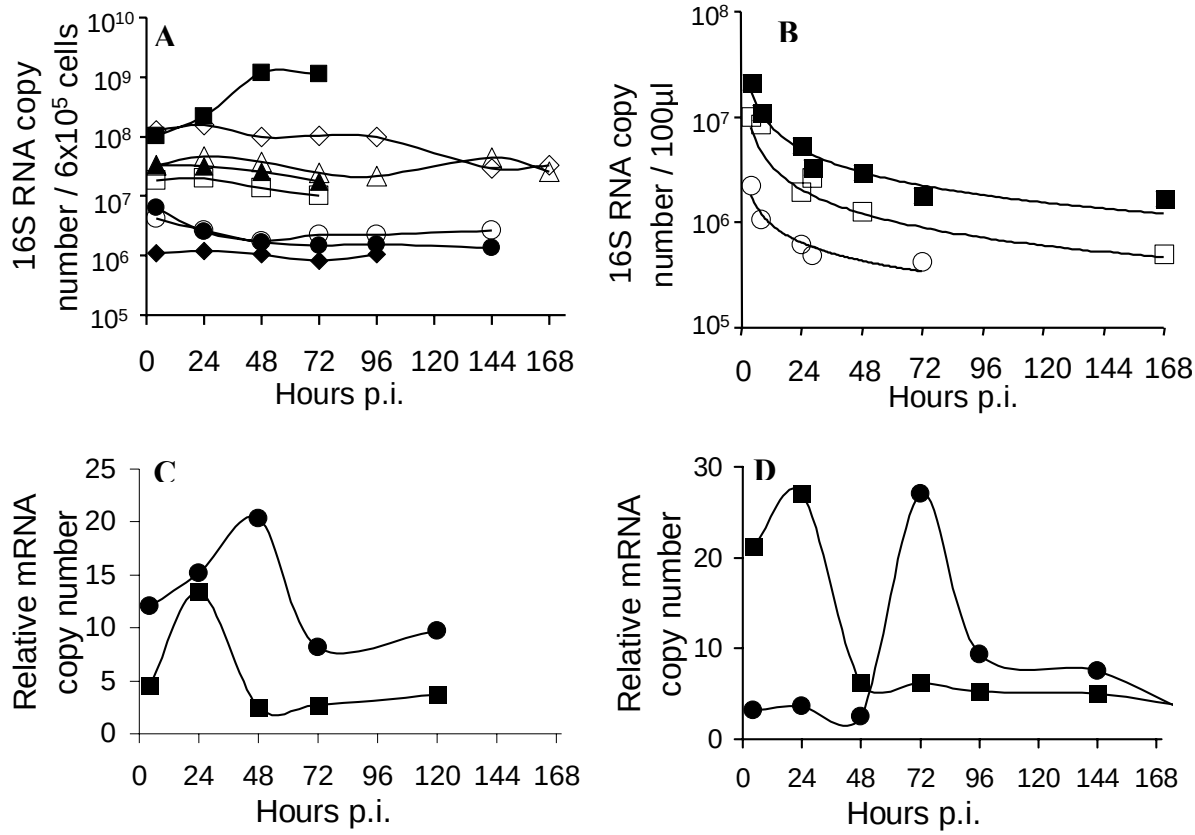

Fig. 4. mRNA levels of *16S rRNA*, *groEL-1* and *omcB* genes in *Cpn*-infected DCs. Immature DCs obtained from 7 donors were infected, then harvested at the indicated time points and the copy number of *16S rRNA* and the relative mRNA copy numbers of *groEL-1* and *omcB* genes were determined. Panel A: The copy number of *16S rRNA* transcripts during the observation period of 7 days after infection, with some individual differences between donors. ■ HEp-2 cells; ● DCs, donor 110; ○ DCs, donor 121; and ♦, Δ, □, ▲, ◊ DCs, 5 additional donors. Panel B: The copy number of *16S rRNA* in 3 dilutions of the bacterium suspension during a 7-day incubation period at 37 ^{o}C without the presence of eukaryotic cells. ■ 1.2 x 10^6 IFU/100 µl; □ 6 x 10^5 IFU/100 µl; ○ 1.2 x 10^5/100 µl bacterium suspension. Panels C (donor 110) and D (donor 121): The relative mRNA copy numbers of *groEL-1* and *omcB* transcripts in the DC populations. ■ *groEL-1* (x 10^5); ● *omcB* (x 10^3).

5.2.4. Production of IFN-γ in DC cultures and the effects of its neutralization

The growth of chlamydiae in different epithelial cell lines is restricted by IFN-γ (92, 97, 113). Moreover, IFN-γ treatment of human monocyte-derived macrophages induced the generation of nonreplicative, morphologically aberrant inclusion bodies and differentially expressed *Cpn* genes that mediate *Cpn* persistence in these cells (1). We speculated that the production of some soluble factors present in the culture medium of human DC populations, contributes to the inhibition of the full bacterial replication in these cells. Thus, we examined whether IFN-γ is produced in the DC cultures after *Cpn* infection. The medium of the DC

cultures was harvested at different times after infection with *Cpn* or a mock preparation. The results showed the presence of IFN-γ in the medium of the DC cultures on days 1-10, with maximum concentration on day 5 after infection (>3000 pg/ml), the concentration subsequently decreasing (Fig. 5).

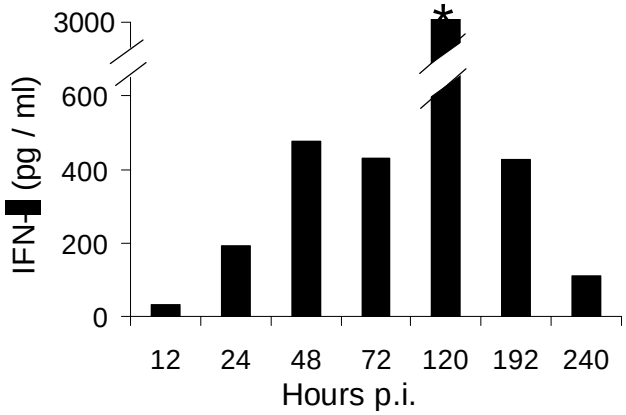

Fig. 5. The concentration of IFN-γ in the culture medium of *Cpn*-infected DCs. The concentration of IFN-γ in the culture medium of *Cpn*-infected DCs harvested at the indicated times was tested in an ELISA. Data indicate the IFN-γ concentrations of DC cultures prepared from a seropositive donor. The IFN-γ concentrations in cultures infected with the mock preparation were below the level of detectability. The experiment was carried out by using 3 additional donors with similar results. ■ IFN-γ produced by DC cultures, * indicates > 3000 pg/ml.

However, the contaminating lymphocytes in the DC cultures were probably stimulated by the DCs presenting the chlamydial peptides, and it was therefore not clear to what extent the DCs or the stimulated contaminating lymphocytes produced the IFN-γ present in the culture medium. Thus, the cellular components of the DC cultures were analyzed for the expression of IFN-γ at different times after infection. Figure 6 shows that, at 12 and 48 h after infection, a higher percentage of the contaminating lymphocytes than DCs expressed IFN-γ. However, the presence of IFN-γ in cells negative for B and T lymphocytes, NK cells, monocytes and macrophage markers, but with the size of DC and expressing HLA-DR and CD11c, a surface antigen specific for human monocyte-derived DCs (17), clearly demonstrated at the single cell level the capacity of a subpopulation of DCs to produce IFN-γ in response to *Cpn* infection.

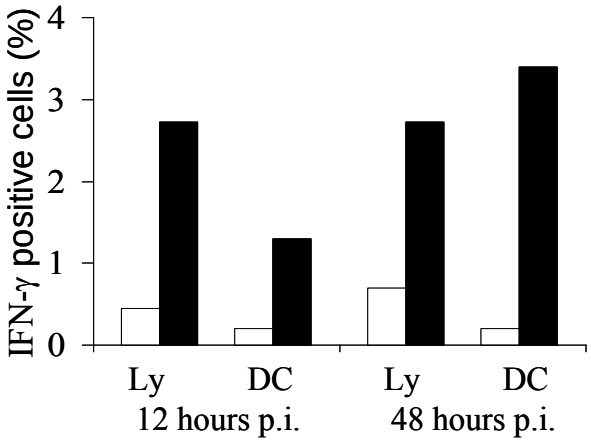

Fig. 6. IFN-γ production by DCs. IFN-γ production by DCs and the contaminating lymphocytes was determined at the single cell level. Cells harvested from the infected and mock-treated DC cultures at the indicated times were stained with the appropriate monoclonal antibodies. The results were read by flow cytofluorometry. DCs were defined in a four-color IF assay and flow cytofluorometry by DC morphology, negativity for the lineage cocktail and the coexpression of CD11c, HLA-DR and IFN-γ. Lymphocytes were defined in a two-color IF assay and flow cytofluorometry by lymphocyte morphology and positivity for the lineage cocktail and the anti-human IFN-γ monoclonal antibody. In the figure the percentage of lymphocytes positive for IFN-γ is reduced 10 fold. The experiment was carried out by using 4 additional donors with similar results. ■ *Cpn*-infected cells; □ mock-infected cells.

To investigate the possible inhibitory effect of various factors, including IFN-γ, produced by the infected DC cultures, the supernatants of the infected DCs either treated or not with neutralizing anti-IFN-γ antibodies were transferred to HEp-2 cells infected with *Cpn*. The supernatants of the *Cpn*-infected DCs without treatment decreased the *Cpn* growth in HEp-2 cells infected with an MOI of 0.1 by up to 168-fold, as compared with that of the mock-infected DCs, whereas the inhibition was only 39-fold in HEp-2 cells receiving supernatants from *Cpn*-infected DC cultures treated with anti-IFN-γ antibodies (not shown). These results indicate an inhibitory effect on *Cpn*-infected HEp-2 cells by cellular factors, including IFN-γ, produced by the infected DC cultures. To test the hypothesis that IFN-γ produced by *Cpn*-infected DC cultures is the factor that inhibits the production of infectious particles, infected DCs were treated with a neutralizing antibody against IFN-γ for 5 days and tested for the presence of infectious EBs on HEp-2 cells. Although the neutralization of IFN-γ was sufficient, *i.e.* the IFN-γ concentration decreased to a level of <9.4 pg/ml in the anti-IFN-γ-treated cultures, as compared with the IFN-γ content of >3000 pg/ml in the untreated cultures on day 5 after infection, no difference in the level of infectious EBs in DCs treated or not with anti-IFN-γ was observed in tests on HEp-2 cells (not shown). These results indicate that the production of IFN-γ is not the major factor that inhibits the growth of

Cpn in the DCs. Since the production of TNF-α by *Cpn*-infected DCs was reported earlier (126), we tested whether treating of these cells with neutralizing anti-TNF-α antibodies (12 μg/ml, Abcam) would increase the production of infectious EBs. Similarly to the results obtained with anti-IFN-γ treatment, it was found that the anti-TNF-α treatment, or the combined treatment with anti-IFN-γ and anti-TNF-α, of infected DCs, did not change the production of infectious progeny by the cells (not shown), suggesting a mechanism(s) different from that of IFN-γ and TNF-α in the persistent state of *Cpn* in the DCs.

Evidence related to the generation of IFN-γ by human DCs is limited (35). We have shown for the first time that *Cpn*-infected human DCs can synthetize IFN-γ. Our results concerning identification of cell types at the single cell level demonstrated IFN-γ production in the contaminating lymphocytes and, more importantly, in human monocyte-derived DCs upon *Cpn* infection. The capability of a small proportion of the DCs to synthetize IFN-γ might have an impact in the early phase of the immune response to intracellular pathogens. For example, IFN-γ obtained from DCs may function as an autocrine amplifier and costimulator of TLR-mediated responses in human DCs, as suggested for the *Mycobacterium*-induced production of IFN-γ by human DCs (35). Our finding that IFN-γ production by human DCs is not restricted to stimulation with *Mycobacterium*, with its complex bacterial structure and replication, but can be induced by other intracellular pathogens, such as *Cpn*, might indicate a more fundamental significance of this phenomenon. In our study, the IFN-γ-producing DCs were characterized as non-T, non-B, non-monocyte/macrophage and non-NK cells by the negativity with the lineage cocktail and positivity for the CD11c and HLA-DR molecules; the DC nature of the identified cells is therefore highly probable. The concentration of IFN-γ measured in the DC culture medium, which reflects the balance of the rates of secretion and degradation of IFN-γ, and the falling viability of the infected cells, indicates a decrease in IFN-γ production after day 5. Since other cytokines, including IL-12, IL-18, type I IFNs and TNF-α, have been reported to be released by human DC cultures upon stimulation (35, 96, 126), the production of IFN-γ by *Cpn* stimulation may be mediated by the presence of these cytokines in the cultures.

5.3. Interactions of DCs with HCMV-Oslo

5.3.1. Lack of replication of HCMV-Oslo in DC populations

At the beginning of our experiments it was not known whether the clinical isolate HCMV-Oslo, passaged in fibroblast cells 10 times, would replicate in iDCs. Thus iDCs were infected at an MOI of 3-5 overnight, as described in Section 4.5 and 4.14. No cytopathic effect was observed on the DCs during the 10-day experiment. Supernatants of infected DCs harvested on days 7 and 10 after infection were inoculated onto MRC-5 cells, without any sign of HCMV infection examined visually for cytopathic effect and for the expression of MIE antigens by using monoclonal antibodies and IF microscopy during the observation period of 14 days. Thus, the replication of HCMV-Oslo was abrogated in iDCs and the blockade of the replication was defined at or before the expression of MIE proteins.

5.3.2. Maturation of DCs following treatment with late conditioning media

Late HCMV conditioning medium containing infectious virus particles as well as viral and cellular products, and applied to the iDCs on day 7, stimulated the expression of the costimulatory molecules CD40 and CD86, the maturation marker CD83 and increased the fluorescence intensity of the expressed HLA-DR molecules on DCs prepared from 4 HCMV seropositive and 2 seronegative donors, as assayed on day 10 (Fig. 7). The stimulation was similar in cultures treated either with TNF-α or with HCMV (not shown), indicating that the factor(s) present in the HCMV conditioning media is a strong inducer for certain costimulatory and CD83 molecules.

It was not clear whether viral particles are necessary for the stimulation. Thus, immature DCs were also treated with late HCMV conditioning medium ultracentrifuged to remove viral particles. The treatment induced elevated levels of maturation molecules on the DCs obtained from seropositive or seronegative donors, indicating that the presence of viral particles is not necessary for the enhanced expression of the DC maturation markers (Fig. 7). Similar increases in the expression of these surface markers were observed on the use of DCs obtained from 4 additional seronegative and 4 seropositive donors. Thus, the stimulatory effect of the virus particles is not involved in the increased expression of maturation markers on DCs.

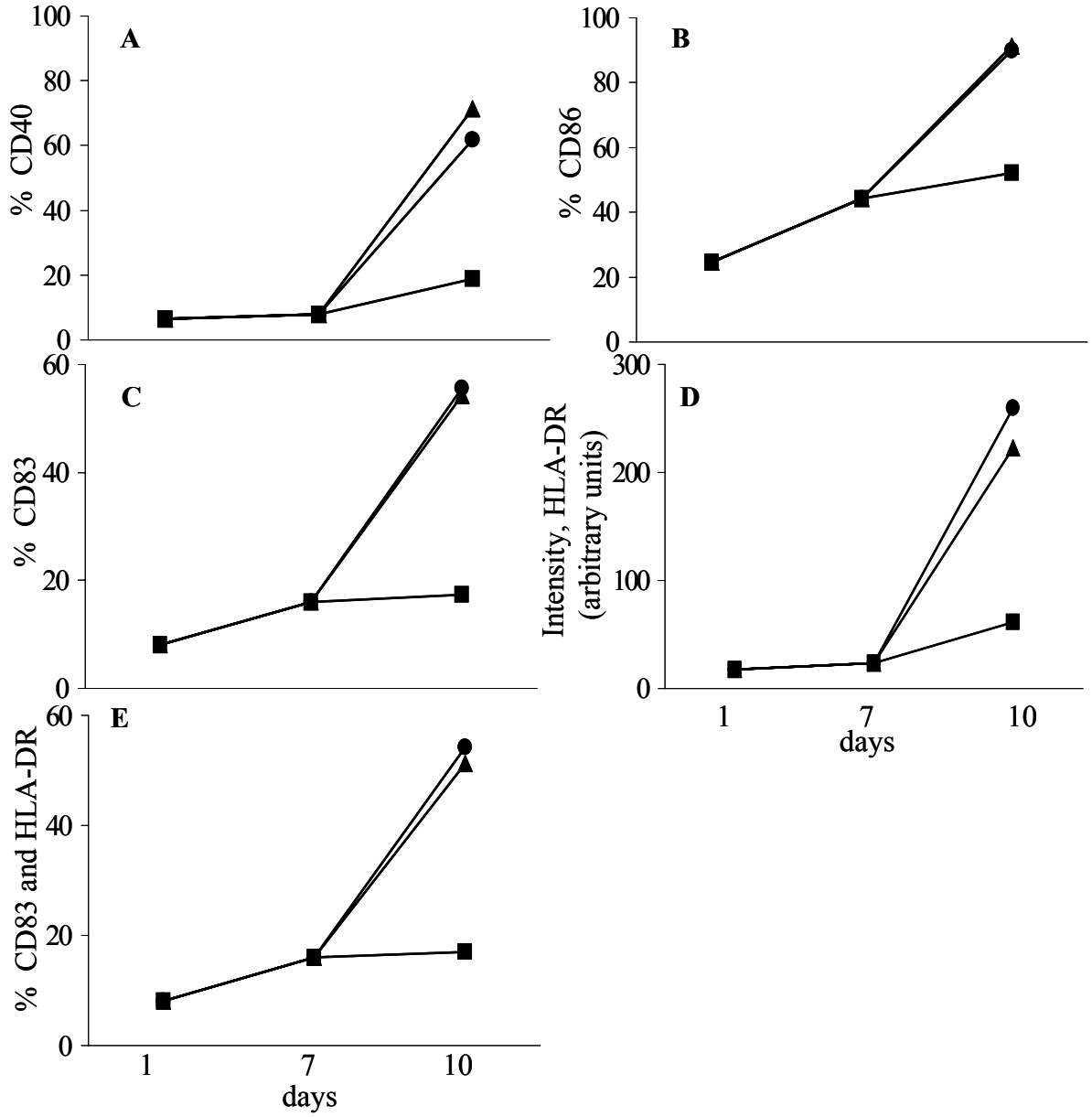

Fig. 7. DC maturation following interaction with late HCMV conditioning medium or with the same medium depleted from virus particles. Immature DCs obtained from a seronegative donor were treated with the late HCMV conditioning medium or the same medium ultracentrifuged to remove viral particles or late mock conditioning medium. The percentages of DCs expressing CD40, CD86 and CD83 surface markers and the intensity of the HLA-DR molecules were determined. Ten-fold reduced values are shown for the fluorescence intensity of HLA-DR molecules (Panels A- D). The results also revealed that all cells expressing the DC maturation marker CD83 were positive for HLA-DR and negative for surface markers of T and B lymphocytes, NK cells and monocytes, indicating the true DC nature of these cells (Panel E). ● late HCMV conditioning medium; ▲ ultracentrifuged late HCMV conditioning medium; ■ late mock conditioning medium.

5.3.3. Maturation of DCs following treatment with early conditioning medium

To compare the effects on DC maturation of the HCMV conditioning medium harvested early or late after infection, media were clarified from cell debris, and then ultracentrifuged to remove virions. Expression levels of the DC maturation markers were increased after treatment with the ultracentrifuged supernatants of the early or late HCMV conditioning medium with no significant differences in the percentages of DCs expressing CD86 (47.5% or 52.4%, respectively, vs. 27.2% or 23.5%, respectively, in mock-treated cultures) and CD83 molecules (38.1% or 44.8%, respectively, vs 9.7% or 12.3%, respectively, in mock-treated cultures) and in the intensity of HLA-DR molecules (1542 or 1600 mean channel values, respectively, vs. 1020 or 1089, respectively, in mock-treated cultures) (Annex 3, Figure 2).

5.3.4. Function of DCs treated with early or late conditioning medium obtained from HCMV-infected fibroblast cells

However, it was an open question as to whether the DCs treated with the early or late HCMV conditioning medium are functionally mature, i.e. they are able to stimulate T lymphocytes. Thus we investigated whether the percentages of IFN-γ–producing T cells would increase following coculturing with DCs which were activated with the HCMV or mock conditioning medium. The iDCs were treated with early or late HCMV or mock conditioning medium for 3 days and incubated with the autologous lymphocytes for 8 h then stained for CD4 and CD8 molecules and measured by cell cytofluorometry. A subset of lymphocytes was directly stimulated with the HCMV or mock conditioning medium without the presence of DCs, and the percentages of CD4+ or CD8+ cells producing IFN-γ were subtracted from the data obtained following the coculturing of lymphocytes with activated DCs. The results (Fig. 8) showed 4.5% CD4+ and 2.2% CD8+ cells producing IFN-γ, when these cells were obtained from HCMV seropositive donors and cocultured with DCs treated with the late HCMV conditioning medium. The DCs treated with the early HCMV conditioning medium or with the ultracentrifuged late HCMV medium failed to induce higher ratios of IFN-γ-producing CD4+ and CD8+ cells of the seropositive donors than DCs treated with the mock medium (not shown), probably because no antigens, i.e, virus particles, dense bodies and viral proteins were present in a sufficient amount in these media. The CD4+ and CD8+ T cells from seronegative donors did not produce IFN-γ in higher proportion when cocultured with DCs treated by late HCMV conditioning medium as

compared to those cocultured with mock medium-treated DCs (Fig. 8). Similar results were obtained in 7 independent experiments using DCs and lymphocytes from 4 seropositive and 3 seronegative donors.

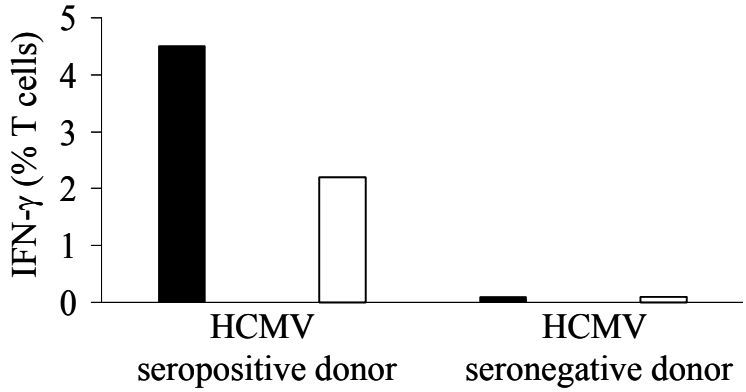

Fig. 8. IFN-γ production of CD4+ and CD8+ lymphocytes cocultured with DCs treated with the late HCMV conditioning medium. Immature DCs were treated with the late HCMV or mock conditioning medium and IFN-γ production of the CD4+ and CD8+ lymphocytes was determined. Data represent percentages of lymphocytes producing IFN-γ after subtracting the percentages obtained by treatment with the late mock preparations. CD4+ lymphocytes: ■; CD8+ lymphocytes: □.

To test the ability of early HCMV conditioning medium to functionally activate DCs, the early HCMV conditioning medium was supplemented with a foreign antigen, such as UV-inactivated *Cpn* (strain TW-183). Bacterial antigens were prepared as described in Section 4.1 and for UV inactivation, *Cpn* suspension was placed under a UV lamp (15W at 30 cm on ice) for 20 min (128)). Early HCMV conditioning medium supplemented with UV inactivated *Cpn* activated DCs to elicit a *Cpn*-specific IFN-γ production in CD4+ (1.3%) and CD8+ (1.2%) cells obtained from *Cpn* seropositive donors, but not in those from *Cpn* seronegative donors (Fig. 9). DCs treated with the mock medium supplemented with the *Cpn* preparation induced a higher ratio of CD4+ (0.6%) and CD8+ (0.7%) cells producing IFN-γ than the mock medium alone (0.1 and 0.05), indicating that the DCs are also stimulated by the *Cpn* preparation itself. The percentage of IFN-γ–producing CD4+ and CD8+ cells was, however, higher when DCs were treated with the combination of early HCMV conditioning medium and *Cpn* preparation, suggesting that the HCMV early medium exerted a functional maturation of DCs (Fig. 9). Similar data were obtained in 4 additional independent experiments.

The functional maturation of DCs upon treatment with the late HCMV conditioning medium was confirmed by induction of HCMV-specific lymphoproliferative responses of T cells (Annex 3).

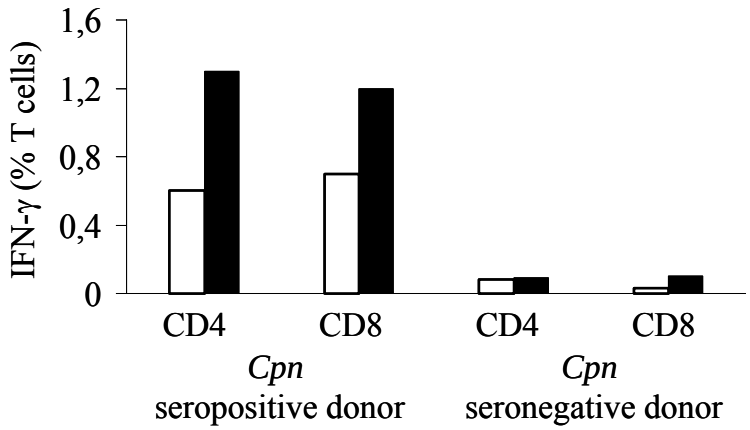

Fig. 9. IFN-γ production of CD4+ and CD8+ lymphocytes cocultured with DCs treated with the early HCMV conditioning medium supplemented with *Cpn* preparation. Immature DCs were treated with the early HCMV or early mock conditioning medium supplemented with a UV-inactivated *Cpn* antigen preparation at a dose of 0.05 IFUs before UV-irradiation, and IFN-γ production of the CD4+ and CD8+ lymphocytes was determined. Data represent percentages of lymphocytes producing IFN-γ after subtracting the percentages obtained by treatment with the mock preparations. Early mock conditioning medium + *Cpn* preparation: □; early HCMV conditioning medium + *Cpn* preparation: ■.

These results indicate that at least 2 distinct components are present in the late conditioning medium: 1. a soluble cellular factor(s) that stimulates the induction of maturation markers on DCs, 2. viral particles and aggregated viral proteins that are internalized, processed and presented by DCs to the autologous lymphocytes. Our results also indicate that soluble cellular factors are similarly present in the early conditioning medium that stimulates the expression of DC maturation markers on the DCs and, when antigen (e.g. inactivated *Cpn* preparation) is supplemented, these DCs induce antigen-specific T lymphocyte responses. These results might represent a form of interactions between *Cpn* and HCMV in individuals chronically infected with both pathogens. It is not clear whether the same or a different factor(s) present in the early and late HCMV conditioning medium is responsible for the induction of the maturation markers on iDCs. Characterization of the viral proteins responsible for the HCMV-specific stimulation of CD8+ cells by the DCs was not the subject of our study, though phosphoprotein 65 (pp65) and the MIE antigens released by lytically infected MRC-5 cells are the major targets for the CTL responses on healthy seropositive individuals (42, 93). Since no virus replication was observed in the infected iDCs, the stimulation of autologous CD8+ cells producing IFN-γ or incorporating BrdU occurred via presentation by DC MHC-I molecules of the exogenous antigens obtained from the late HCMV conditioning medium. In an earlier study, the percentage of DCs expressing CD83 was high when they were treated with infected cell

conditioning medium obtained at 24h, and lower when they were treated with medium obtained at 48-72 h after infection, and the secretion of transforming growth factor β1 was suggested to be responsible for the inhibition of the expression of CD83 by the late medium. HCMV conditioning medium obtained later than 72 h post infection was not investigated in this study (4). Our results do not support the inhibition of the expression of CD83 by the late medium obtained at 7-9 days post infection, indicating that up- and down-regulatory factors might be released in a special temporal pattern onto the supernatants of infected MRC-5 cells, thus the concentration of these factors may be different in the supernatants at various times after infection. In addition, the duration of the coincubation of DCs with the conditioning medium may modulate the effect of these factors on the maturation of DCs; in the previous study (4) a 1-day, in our experiments a 3-day coincubation time was used. Our results are in contrast with the downregulation of maturation markers and impaired DC function following infection with fibroblast-adapted HCMV strains (9). The reason for the contradictory results may be connected with the virus preparation applied for the infection/treatment of the iDCs. Beck et al. (9) used ultracentrifuged virus pellets for treatment, whereas we utilized the culture medium obtained from the infected fibroblasts, or the ultracentrifuged supernatant depleted from virus particles. The differences in the results may indicate various responses of DCs to different components or products of certain pathogens (57).

6. Conlusion

The novel observations presented in this thesis are the following:

Chlamydophila pneumoniae growth in mice following repeated infection:

- quantitative measurement of *Cpn* DNA not only in the lungs but also in the peripheral blood in high copy numbers soon after the primary and repeated inoculations, and with a decreasing copy number at later time points, but DNA was still detectable in both the lungs and the blood a long period after infection in a small number of mice.

Interaction of DCs with *Chlamydophila pneumoniae*:

- the localization of *Cpn* antigens in CD83 positive and negative, CD86 positive and negative, and HLA-DR positive subpopulations of DCs during the maturation process;
- the expression of mRNA transcripts of the *16S rRNA*, *groEL-1* and *omcB* genes, but a low rate of mRNA expression of the *ftsK* gene in DC cultures, as determined by quantitative real-time PCR, indicating an active, though restricted bacterial metabolism in the DCs;
- the production of IFN-γ by a subpopulation of DCs exposed to *Cpn* infection, which might contribute to the immune responses to *Cpn*, or to the immunopathology of *Cpn*-associated chronic diseases.

Interaction of DCs with HCMV-Oslo:

- the factor(s) responsible for the upregulated expression of maturation markers on DCs is not only present in the early HCMV conditioning medium as described earlier (4), but is also released by the infected MRC-5 cells late, between days 7 and 9 after HCMV infection;
- the soluble factor(s) produced late after infection of MRC-5 cells is distinct from viral particles and protein aggregates with the size sedimented at 100 000g for 1 h;
- the late HCMV conditioning medium containing the cellular soluble factor(s) and the virus particles and viral proteins induces both the expression of maturation markers and the antigen processing/presentation function of the DCs;
- the DCs treated with the early HCMV conditioning medium are functionally mature, as demonstrated by supplementing this medium with an antigen, such us inactivated *Cpn* bacterium or its components.

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Annex

1. **Kis Z**, Burian K, Treso B, Acs K, Prohaszka Z, Fust G, Gonczol E, Endresz V. Inflammatory- and immune responses in relation with bacterial replication in mice following re-infections with *Chlamydophila pneumoniae* Inflamm Res, accepted. **IF.: 2.281**

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