Simvastatin Reduces *Chlamydia pneumoniae*–Mediated Histone Modifications and Gene Expression in Cultured Human Endothelial Cells

Bernd Schmeck,* Wiebke Beermann,* Philippe Dje N’Guessan, Andreas C. Hocke, Bastian Opitz, Julia Eitel, Quoc Thai Dinh, Martin Witzenrath, Matthias Krüll, Norbert Suttorp, Stefan Hippenstiel

**Abstract**—Inflammatory activation of the endothelium by *Chlamydia pneumoniae* infection has been implicated in the development of chronic vascular lesions and coronary heart disease by seroepidemiological and animal studies. We tested the hypothesis that *C pneumoniae* induced inflammatory gene expression is regulated by Rho-GTPase–related histone modifications. *C pneumoniae* infection induced the liberation of proinflammatory interleukin-6, interleukin-8, granulocyte colony-stimulating factor, macrophage inflammatory protein-1β, granulocyte/macrophage colony-stimulating factor, and interferon-γ by human endothelial cells. Cytokine secretion was reduced by simvastatin and the specific Rac1 inhibitor NSC23766 but was synergistically enhanced by inhibitors of histone deacetylases trichostatin A and suberoylanilide hydroxamic acid. Infection of endothelial cells with viable *C pneumoniae* but not exposure to heat-inactivated *C pneumoniae* or infection with *Chlamydia trachomatis* induced acetylation of histone H4 and phosphorylation and acetylation of histone H3. Pretreatment of *C pneumoniae*-infected cells with simvastatin or NSC23766 reduced global histone modifications as well as specific modifications at the il8 gene promoter, as shown by chromatin immunoprecipitation. Reduced recruitment of nuclear factor κB p65/RelA as well as of RNA polymerase II was observed in statin-treated cells. Taken together, Rac1-mediated histone modifications seem to play an important role in *C pneumoniae*-induced cytokine production by human endothelial cells. *(Circ Res. 2008;102:888-895.)*

**Key Words:** endothelial cells ■ cytokines ■ statins ■ *Chlamydia pneumoniae* ■ histones

*Chlamydia* are Gram-negative bacteria that are obligate intracellular parasites of eukaryotic cells, including endothelial cells. Although chronic or recurrent infections with *Chlamydia pneumoniae* have been associated with the development of vascular lesions and atherosclerosis, little is known about the molecular pathogenesis. Infection of endothelial cells with *C pneumoniae* may initiate and perpetuate local inflammation by inducing cytokine release (eg, interleukin [IL]-6, IL-8) and adhesion molecule expression (P-/E-selectin, intercellular adhesion molecule-1, vascular cell adhesion molecule-1), which subsequently results in recruitment of inflammatory cells to the endothelium.

Infection of the endothelium by *Chlamydia* is recognized by different pattern-recognition receptors. Membrane-bound Toll-like receptors TLR2 and TLR4 have been demonstrated to mediate host defense against *C pneumoniae* or chlamydial components. In addition, the nucleotide-binding oligomerization domain protein NOD1 was identified as part of a cytosolic surveillance system detecting intracellular Chlamydia in human endothelial cells. The detection of pathogens by these pattern-recognition receptors resulted in the activation of complex signaling pathways, including the stimulation of nuclear factor (NF)-κB–dependent gene transcription.

Increasing evidence indicates that histone modifications may serve as combinatorial code for the transcriptional activity state of genes in many cellular processes by loosening the DNA–histone interaction and unmasking of transcription factor binding sites. In chromatin, 146 base pairs of DNA are wrapped 1.65 turn around a histone octamer (H2A, H2B, H3, H4). Transcription repression or gene activation is regulated by specific covalent modifications of accessible N-terminal histone tails, including acetylation (mostly lysine), phosphorylation (serine/threonine), and methylation (lysine). Phosphorylation at Ser10 on H3 and acetylation at Lys14 of H4 seems to have a special impact on gene regulation. These modifications were implicated in lipopolysaccharide (LPS)-stimulated activation of dendritic...
cells,\(^1\)\(^9\) as well as in *Listeria monocytogenes*–induced activation of human endothelial cells.\(^2\)\(^0\)

In this study, we tested the hypothesis that *C pneumoniae* regulated inflammatory gene expression in endothelial cells by histone modifications. *C pneumoniae* induced expression of IL-6, IL-8, granulocyte colony-stimulating factor (G-CSF), macrophage inflammatory protein (MIP)-1\(\beta\), granulocyte/macrophage colony-stimulating factor (GM-CSF), and interferon (IFN)-\(\gamma\) was reduced by simvastatin and the Rac1 inhibitor NSC23766 but was synergistically enhanced by inhibitors of histone deacetylases (HDACs). Pretreatment of cells infected with *C pneumoniae* with simvastatin or NSC23766 reduced global histone modifications, as well as specific modifications at the *il8* gene promoter. Moreover, reduced recruitment of NF-\(\kappa\)B p65/RelA as well as of RNA polymerase II (Pol II) was observed in statin-treated cells. Taken together, Rac1-mediated histone modifications seem to play an important role in *C pneumoniae*–induced cytokine production by human endothelial cells.

**Materials and Methods**

Human umbilical vein endothelial cells (HUVECs) and human aortic endothelial cells (HAECs) were infected by *C pneumoniae* strain CWL 029 or *Chlamydia trachomatis* serovar K. Cytokine release was measured by ELISA and Bioplex Protein Array system. Histone modifications were analyzed globally by Western blot and on the promoter level by chromatin immunoprecipitation (ChIP). Details regarding reagents and methodology are provided in the online data supplement, available at http://circres.ahajournals.org.

**Results**

*C pneumoniae*–Induced Cytokine Expression Was Inhibited by Simvastatin and Rac1 Inhibitor NSC23766

*C pneumoniae* infection of the endothelium is suspected to contribute to the initiation or progression of atherosclerosis,\(^3\)\(^,\)\(^2\)\(^1\)\(^–\)\(^2\)\(^5\) and cytokine release of infected endothelial cells may promote inflammation of the vessel wall.\(^2\)\(^3\)\(^,\)\(^2\)\(^6\)\(^,\)\(^2\)\(^7\) As shown in Figure 1A, infection of HUVECs with *C pneumoniae* CWL (5 multiplicities of infection [mois]) for 24 hours induced the expression of IL-6, IL-8, G-CSF, GM-CSF, MIP-1\(\beta\), and IFN-\(\gamma\). Infection with *C trachomatis* did not induce IL-8 or GM-CSF (Figure 1B; 5 mois). However, infection with *C pneumoniae* did not induce significant release of IL-2, IL-4, IL-7, IL-10, IL-12(p70), and IL-13 by endothelial cells in our experimental setup (data not shown).

To analyze whether only viable bacteria induce cytokines, we exposed cells to viable or heat-inactivated *C pneumoniae* and measured release of cytokine IL-8 and growth factor
GM-CSF. Induction of both factors by heat-inactivated bacteria was significantly lower (Figure 1B). Because both heat-inactivated C pneumoniae and viable C trachomatis did not induce significant expression of LPS-inducible IL-8 and GM-CSF in endothelial cells (Figure 1B), cell activation by viable C pneumoniae seems to be independent of LPS, which could be present in the given experimental setting.

The beneficial effects of clinically used statins, which blocked HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A; EC 1.1.1.34) depended, at least in part, on the inhibition of small GTP-binding Rho protein family members.28,29 Pretreatment of endothelial cells with simvastatin for 24 hours (15 μg/mL) or the Rac1-specific inhibitor NSC23766 for 24 hours (200 μmol/L) abolished C pneumoniae–related release of the analyzed cytokines (Figure 1). By using the important chemotactic cytokine IL-8 as a model, we demonstrated the dose dependency of the simvastatin (Figure 2A) and NSC23766 (Figure 2B) effect with respect to C pneumoniae–induced IL-8 release in HUVECs. Thus, inhibition of small GTP-binding Rho proteins reduced C pneumoniae-related cytokine expression in HUVECs.

C pneumoniae, but Not C trachomatis, Induced Histone Modifications in Endothelial Cells

Increasing evidence indicates that the transcription of inflammatory genes is regulated by specific covalent modifications of N-terminal histone tails at Ser10 on H3 (phosphorylation) and at Lys14 of H4 (acetylation).14,19,20 Analysis of HUVECs infected with C pneumoniae for 60 minutes demonstrated rapid induction of global acetylation of H4 and phosphorylation/acetylation of H3 at Ser10/Lys14 starting after 30 minutes of infection, as demonstrated by Western blot (Figure 3A). In addition, the HDAC inhibitor trichostatin A (TSA) (0.01 ng/mL, 60 minutes) induced acetylation of H4 and, to a lesser extent than C pneumoniae, of H3 (Figure 3A).

To test whether this is a C pneumoniae–specific effect, we compared the effect with C trachomatis–induced histone alterations. However, only C pneumoniae induced histone H3/H4 modifications (Figure 3B). Furthermore, heat-inactivated C pneumoniae did not induce the modification of histones in endothelial cells (Figure 3B). To ensure intracellular infection of endothelial cells, HUVECs were exposed to viable or heat-inactivated C pneumoniae or C trachomatis (both, 5 mois). After maturation (72 hours), cells were scraped, briefly sonicated, and

---

**Figure 2.** C pneumoniae–induced IL-8 release was dose-dependently blocked by simvastatin or specific Rac1 inhibition. HUVECs were preincubated for 24 hours with the indicated doses of simvastatin (A) or the specific Rac1 inhibitor NSC23766 (B) and infected with 5 mois of C pneumoniae CWL for 15 hours. Cytokine release was measured in the supernatant by ELISA. Data are shown as means ± SEM of at least 3 independent experiments. *P<0.05 compared with uninfected control cells; #P<0.05 compared with infected cells without inhibitors.

**Figure 3.** Viable C pneumoniae, but not heat-inactivated bacteria or C trachomatis, time-dependently induced phosphorylation/acetylation of H3 and acetylation of H4. A, HUVECs were infected with C pneumoniae (5 mois) for the indicated time periods or exposed to TSA (0.01 ng/mL, 60 minutes). Histone modifications were detected by Western blot using antibodies specifically detecting Ac-H4 or phosphorylated/acetylated (Ser10/Lys14) H3. B, HUVECs were exposed to viable C pneumoniae (CWL), heat-inactivated C pneumoniae (hiCWL), or C trachomatis serovar K (CTK), each with 5 mois, for 60 minutes. Histone modifications were detected by Western blot using antibodies specifically detecting Ac-H4 or phosphorylated/acetylated (Ser10/Lys14) H3. Representatives of 3 independent experiments are shown. C, HUVECs were exposed to viable or heat-inactivated C pneumoniae or C trachomatis (both, 5 mois). After maturation (72 hours), cells were scraped, briefly sonicated, passedaged (1:3) onto HEP-2 cells seeded on glass coverslips, and visualized by confocal microscopy (red, F-actin; green, bacteria).
passaged (1:3) onto HEp-2 cells seeded on glass coverslips. Only in HEp-2 cells exposed to HUVEC extracts with viable C pneumoniae, but not to cells incubated with heat-inactivated C pneumoniae, an intracellular LPS signal could be detected (Figure 3C). Because HEp-2 cells do not efficiently endocytose LPS, C pneumoniae infection was demonstrated in concordance with the literature.11,31

Inhibition of HDACs Increased C pneumoniae–Related Cytokine Expression

To test whether C pneumoniae–related histone modifications are of functional importance for the observed cytokine induction, cells were infected with a low moi (0.5) of C pneumoniae and exposed to low doses of HDAC inhibitors, which did not induce cytokine expression per se (Figure 4). Exposure of endothelial cells to HDAC inhibitor TSA (0.1 ng/mL) and to C pneumoniae (0.5 moi) enhanced the expression of IL-6, IL-8, G-CSF, GM-CSF, MIP-1β, and IFN-γ (Figure 4). Moreover, a chemically unrelated HDAC inhibitor, suberoylanilide hydroxamic acid (SAHA),32 also induced C pneumoniae–related IL-8 expression in HUVECs (Figure 4). Furthermore, exposure of endothelial cells to both HDAC inhibitors significantly increased the C pneumoniae–induced IL-8 release. Thus, histone acetylation seems to contribute to C pneumoniae–related cytokine release in human endothelial cells in vitro.

To analyze whether histone acetylation is only involved in cytokine induction by viable bacteria, we exposed cells to viable or heat-inactivated C pneumoniae. IL-8 induction by heat-inactivated bacteria was significantly lower but could also be enhanced by TSA (Figure 5D). The same results were obtained with respect to GM-CSF induction (data not shown).

Figure 4. HDAC inhibition synergistically enhanced C pneumoniae–induced cytokine release. HUVECs were preincubated with the HDAC inhibitor TSA (0.01 ng/mL) and then infected with C pneumoniae (0.5 moi) for 15 hours. Release of cytokines was measured in the supernatant by Bioplex assay. Data are shown as means±SEM of at least 3 independent experiments. *P<0.05 from C pneumoniae– and TSA-exposed cells.

Figure 5. HDAC inhibitors TSA and SAHA synergistically enhanced C pneumoniae–induced IL-8 release by human umbilical vein or aortic endothelial cells. HUVECs (A) or HAECs (B/C) were preincubated with HDAC inhibitors SAHA (1 nmol/L) (A and C) or TSA (0.01 ng/mL) (B) and then infected with C pneumoniae (0.5 moi) for 15 hours. Release of IL-8 was measured in the supernatant by ELISA. Data are shown as means±SEM of at least 3 independent experiments. *P<0.05 from C pneumoniae– and inhibitor-exposed cells. D, HUVECs were preincubated with the HDAC inhibitor TSA (0.01 ng/mL) (B) and then infected with viable or heat-inactivated C pneumoniae (hiCWL) (both, 5 moi) for 15 hours. Release of IL-8 was measured in the supernatant by ELISA. Data are shown as means±SEM of at least 3 independent experiments. *P<0.05 comparing viable and heat-inactivated C pneumoniae; #P<0.05 from heat-inactivated C pneumoniae with or without preincubation with TSA.

C pneumoniae Induced Histone Alterations at the il8 Gene Promoter

To gain more insight into the impact of histone modification on C pneumoniae–related cytokine regulation, we analyzed IL-8 expression as a model at promoter level by applying ChIP. Infection of endothelial cells with C pneumoniae induced rapid acetylation of H4 and phosphorylation/acety-
lation of H3 at Ser10/Lys14 at the il8 promoter (Figure 6A). Moreover, we observed recruitment of NF-κB p65/RelA subunit to the il8 promoter in infected cells, which is known to be essential for IL-8 expression.33 In addition, binding of Pol II at the il8 promoter further indicates the start of gene transcription. As expected from results in Figures 3B and 5D, heat-inactivated C pneumoniae induced no modification of analyzed histone residues and only weak recruitment of p65 and Pol II to the il8 promoter in ChIP analysis (Figure 6B).

Simvastatin Reduced C pneumoniae–Induced Global and Gene-Specific Histone Modifications
Because simvastatin and Rac1 inhibitor NSC23766 both inhibited C pneumoniae–related cytokine production, we assessed their effect on global histone modification and on gene-specific (IL-8) regulation. As can be seen in Figure 7A, preincubation with both simvastatin and NSC23766 reduced global acetylation of H4 and phosphorylation/acetylation of H3 at Ser10/Lys14. ChIP experiments (Figure 7B) showed that simvastatin reduced the C pneumoniae–related acetylation of H4 and phosphorylation/acetylation of H3 at Ser10/Lys14 at the il8 promoter. In parallel, recruitment of p65, as well as of Pol II, was abolished back to control levels in simvastatin-exposed endothelial cells, further suggesting that

Discussion
The study presents evidence that statins modulate C pneumoniae–induced inflammatory activation of human endothelial cells by reducing infection-related histone modifications. Viable C pneumoniae–induced expression of proatherosclerotic cytokines IL-6, IL-8, G-CSF, MIP-1β, GM-CSF, and IFN-γ was reduced by simvastatin and the Rac1 inhibitor NSC23766 but was synergistically enhanced by inhibitors of HDACs. Pretreatment with simvastatin or NSC23766 reduced global C pneumoniae–induced histone modifications, as well as specific modifications at the il8 gene promoter. Moreover, reduced recruitment of NF-κB p65/RelA and Pol II was observed in statin-treated cells. Although C trachomatis infected endothelial cells, they did neither induce cytokine expression nor histone modifications.

Impaired endothelial function is observed in a variety of pathological conditions, such as hypertension and atherosclerosis,34,35 and the endothelium is a primary target in several infectious diseases.26 In particular, C pneumoniae infection
has been suggested as a trigger of endothelial inflammation, promoting vascular diseases. In our model, *C. pneumoniae* infection induced the release of chemoattractants, proinflammatory cytokines, as well as myeloid growth factors, by primary human endothelial cells, whereas *C. trachomatis* infected endothelial cells but did not induce cytokine release (this study and Krüll al18).

These cytokines are also thought to play important roles in atherosclerosis: in aortic arches of atherosclerotic mice, expression of monocyte chemoattractant protein-1 and MIP-1β was plaque progression dependent increased. Monocyte chemoattractant protein-1 and also IL-6 were elevated in patients with myocardial infarction and unstable angina. In the Edinburgh Artery study, IL-6 showed a stronger independent predictive value for atherosclerosis and its progression than C-reactive protein or soluble adhesion molecules. Accordingly, IL-6 was associated with significantly increased risk for cardiovascular disease and was found to be the strongest independent biomarker for coronary artery disease–related death in a study with more than 1000 patients. IL-6 and IL-8 were significantly increased in infarct-related coronary artery thrombi and atherosclerotic plaque specimens obtained with a transluminal extraction catheter from cases of acute myocardial infarction. Moreover, GM-CSF was demonstrated to be an important factor in oxidized LDL–induced macrophage proliferation, and Gupta et al found that IFN-γ potentiated atherosclerosis in ApoE knockout mice.

Because release of IL-6, IL-8, and IFN-γ was also observed in *C. pneumoniae*–infected macrophages, these molecules may play an important role in the host defense against *C. pneumoniae*. For example, IFN-γ was demonstrated to impair *C. pneumoniae* infection in macrophages, and mouse models deficient for the IFN-γ receptor showed the higher sensitivity to *C. pneumoniae*. Recent studies imply that histone modifications control eukaryotic gene transcription by effecting transcription factor binding and promoter transactivation. Tight wrapping of DNA around histone octamers appears to obstruct binding of the transcription machinery. Acetylation and phosphorylation of histones change the polarity of histone tails, thereby facilitating uncoiling of DNA and binding of transcription factors and the basal transcription machinery. Viable *C. pneumonia*, but not heat-inactivated bacteria or *C. trachomatis*, induced acetylation of histone H4 and phosphorylation and acetylation of H3. Stimulus-induced phosphorylation of H3 at Ser10 has been reported to be associated with the activation of promoters of mammalian immediate–early genes. Some evidence has been provided that H3 phosphorylation at Ser10 may have a role in the regulation of transcription by acting as a signal for subsequent acetylation of lysines and, in particular, histone H3 Lys14, . Moreover, Agalioti et al demonstrated that both phosphorylation/acetylation (Ser10/Lys14) of histone H3 and acetylation of Lys8 at histone H4 were necessary for the recruitment of general transcription factors and hence for gene transcription. In this line, we provided evidence that histone acetylation is necessary for *L. monocytogenes*–related release of IL-8, but not IFN-γ, by human endothelial cells. Here, we show that *C. pneumoniae*–induced release of both cytokines was enhanced by inhibition of histone deacetylation in human venous and arterial endothelial cells pointing to pathogen-specific gene regulation. Heat-inactivated *C. pneumoniae* were found to induce lower IL-8 and GM-CSF expression, as well as histone modification and p65-/Pol II recruitment to the il8 promoter, suggesting that surface structures of *C. pneumoniae* can activate HUVECs in high concentrations, but for full histone modification, additional effects of viable *C. pneumoniae* seem to be necessary.

Autocrine effects on the histone modification analyzed seemed to be unlikely, because the addressed modifications were observed as early as 30 minutes after stimulation. Furthermore, stimulation of HUVECs with high concentrations of recombinant human IL-8 (50 ng/mL) or tumor necrosis factor-α (50 ng/mL) did not result in comparable histone modifications (data not shown).

The proinflammatory and chemotactic cytokine IL-8 potently recruits leukocytes to sites of infection, thereby contributing to local tissue inflammation. The IL-8 gene promoter has been described previously as being regulated by histone phosphorylation and acetylation. Modifications of both histones H3 and H4 were observed in overall chromatin analysis and were also induced by *C. pneumoniae* infection specifically at the il8 gene promoter, together with recruitment of NF-κB/p65 and Pol II. As Agalioti et al have shown, phosphorylation of H3 at Ser10 and acetylation of H3-Lys14 and H4-Lys8 were followed by recruitment of bromodomain-containing factors TFIIID and SWI/SNF and subsequent transcription. Therefore, our observations, together with previous studies, implicate a sequence of *C. pneumoniae*–related histone phosphorylation, histone acetylation, chromatin remodeling, and successful gene transcription. It has been suggested that the beneficial effects of HMG-CoA reductase–inhibiting statins on endothelial and vascular function depended not only on cholesterol reduction but also on blocking of geranylgeranylation, thereby inhibiting small GTP-binding Rho protein–dependent pathways. Rho-GTPases act as molecular switches in important signaling pathways, including IL-1β–, tumor necrosis factor-α, TLR2, and TLR4–related cell activation. By using simvastatin and the Rac1-specific inhibitor NSC23766, we found an inhibition of *C. pneumoniae*–related cytokine release by both agents, thereby blocking major inflammatory and chemotactic endocrine pathways.

However, further studies are needed to explore the effect of *C. pneumoniae* on the overall gene expression response in *C. pneumoniae* infection in the presence or absence of statins. In addition, release of possible anti- and proinflammatory molecules not detected by, eg, global gene expression analysis (such as nitric oxide, prostacyclin) should be investigated as well.

Accordingly, we demonstrated that simvastatin and the specific Rac1 inhibitor blocked *C. pneumoniae*–induced acetylation of H4 and phosphorylation and acetylation of H3. At the il8 promoter, simvastatin inhibited *C. pneumoniae*–related histone modifications, as well as NF-κB/p65 and Pol II recruitment. In accordance, RhoA and Rac1 activation was shown recently in *C. pneumoniae*–infected type II lung epithelial cells, as well as in vascular smooth muscle cells.
suggesting an important role of Rho protein–related cell activation in C pneumoniae pathogenesis. Taking our observations into account, (simvastatin-related Rac1 inhibition seems to reduce C pneumoniae–related inflammatory activation of (endothelial) cells.

The expression of important proinflammatory mediators is controlled by activation of the transcription factor NF-κB. Because Rac1 was found to be involved in NF-κB activation by C pneumoniae (this study and Dechend et al). heat-inactivated Staphylococcus aureus, and viable S pneumoniae Rac1 may act as a central molecular switch in regulating NF-κB–related gene transcription in bacterial infections.

C trachomatis infected endothelial cells without initiating any detectable cytokine response with respect to the used colony-forming unit and the time frame analyzed. In addition, in contrast to C pneumoniae, C trachomatis did not induce the phosphorylation of mitogen-activated protein kinases or upregulation of adhesion molecule intercellular adhesion molecule-1 in endothelial cells. Although it could not be ruled out that replication of C trachomatis was less sufficient in endothelial cells (which are not their primary target cells), these data suggest that the infection process per se and the formation of a Chlamydia-containing organelle is not sufficient to activate endothelial cytokine release.

Initiation of the endothelial cytokine response against C pneumoniae depends, at least in part, on their recognition by transmembranous and cytosolic pattern-recognition receptors,7–11 and Rac1-related and Rho proteins are considered to act as molecular switches in TLR2 and TLR4 signaling. Therefore, it seems likely that the effects of simvastatin and NSC237666 observed depended on the inhibition of Rho-GTPase–related host cell signaling pathways initiated after recognition of C pneumoniae by pattern-recognition receptors.

In conclusion, we provide evidence that Rac1-related histone modifications contribute to the control of the release of important chemokines in C pneumoniae–infected human endothelial cells.

Acknowledgments

The excellent technical support of Doris Stoll and Jacqueline Hellwig is greatly appreciated. Parts of this work will be included in the doctoral thesis of Wiebke Beermann.

Sources of Funding

This work was supported by Deutsche Forschungsgemeinschaft grants SPP-Kr 2197/1-2 (to M.K. and N.S.) and HU-829/6-1 (to S.H.) and by the Bundesministerium für Bildung und Forschung (BMBF) (to B.S., N.S., and S.H [BMBF-Competence network CAPNETZ, and FORSYS-Partner]).

Disclosures

None.

References


Simvastatin Reduces *Chlamydia pneumoniae*–Mediated Histone Modifications and Gene Expression in Cultured Human Endothelial Cells

Bernd Schmeck, Wiebke Beermann, Philippe Dje N'Guessan, Andreas C. Hocke, Bastian Opitz, Julia Eitel, Quoc Thai Dinh, Martin Witzenrath, Matthias Krüll, Norbert Suttrop and Stefan Hippenstiel

*Circ Res.* 2008;102:888-895; originally published online February 28, 2008; doi: 10.1161/CIRCRESAHA.107.161307

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2008 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/102/8/888

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2008/02/28/CIRCRESAHA.107.161307.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation Research* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Circulation Research* is online at:
http://circres.ahajournals.org/subscriptions/
EXPANDED MATERIALS AND METHODS

Chlamydia strains

*Chlamydia pneumoniae* strain CWL 029 (ATCC, Rockville, USA, VR2282) was cultured and purified as described\(^1\). Briefly, CWL was grown to high titers in cyclohexamide-treated HEp-2 cells. Infected monolayers were harvested from culture flasks and sonicated for 30 s. Cellular debris was removed by centrifugation at 500 x g for 10 min at 4°C. Aliquots of purified elementary bodies were stored at -75°C until used as described\(^1\). For some experiments, *C. pneumoniae* were inactivated by heat treatment (90 °C, 30 min). *Chlamydia trachomatis* serovar K (CTK) was a friendly gift of J. G. Kuipers (Dept. Rheumatology, Medical School Hannover, Germany)\(^2\).

Infection of HUVEC, and HAEC cells

Human aortic endothelial cells (HAEC) were obtained from Clonetics (San Diego, CA), and human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical cord veins. Both cell types were cultured as described previously\(^2\). HUVEC or HAEC were inoculated with *C. pneumoniae* or *C. trachomatis* using a multiplicity of infection (MOI) as indicated in MCDB131 or EGM-2, respectively. Plates were centrifuged at 800 g at 37°C for 1 h and subsequently incubated as indicated.

Confocal laser scanning microscopy (CLSM) analysis of HUVEC and HEK293 cell infection

To ensure intracellular infection of endothelial cells, HUVEC were exposed to viable or heat-inactivated *C. pneumoniae* or *C. trachomatis* (both MOI=5). After maturation (72 h), cells were scraped, briefly sonicated and passaged (1:3) onto HEp-2 seeded on glass coverslips. Cells were fixed with 3% PFA for 20 min and permeabilized with 1% Triton for 15 min as described\(^2\). For chlamydia staining, the primary Ab (a genus-specific monoclonal antibody, “chlamydia culture conformation system”, Sanofi Diagnostics Pasteur, Freiburg, Germany)
was used and subsequently bound antibodies were detected with anti FITC Alexa-488-conjugated goat Ab (4°C, overnight; Molecular Probes, Leiden, Netherlands). Endothelial F-actin was counterstained using Alexa-546-conjugated phalloidin (Molecular Probes). Cells were analyzed using a Pascal 5 confocal laser-scanning microscope (CLSM; Zeiss, Jena Germany).

**Cytokine measurement**

IL-8 concentrations in the supernatants of infected HUVEC or HAEC were quantified using a commercially available sandwich-ELISA Kit (R&D Systems, Wiesbaden, Germany). For cytokine array, after incubation, supernatants were collected and cytokines were analyzed with the Bioplex Protein Array system (BioRad, Hercules, CA) using specific beads, according to the manufacturer´s instructions.

**Chromatin Immunoprecipitation**

Briefly, HUVECs were stimulated and ChIP was performed as described previously with antibodies from Upstate, Waltham, MA (Ac-H4 and P-Ser10/Ac-Lys14-H3), and Santa Cruz Biotechnology, Santa Cruz, CA (p65, Polymerase II). Equal amounts of input DNA was controlled by gel electrophoresis.

The following promoter-specific primers were used: IL-8 sense 5’–AAG AAA ACT TTC GTC ATA CTC CG–3’, antisense 5’–TGG CTT TTT ATA TCA TCA CCC TAC–3’.

**Western blotting**

For histone analysis, cells were lysed in lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 1.5 mM PMSF, 0.5 mM DTT) and H₂SO₄ was added to a final concentration of 0.2 M as described. Protein was precipitated with 50% trichloroacetic acid in a final
concentration of 20%, subjected to SDS-PAGE and blotted on Hybond-ECL membrane (Amersham Biosciences, Freiburg, Germany). Immunodetection was carried out with antibodies specifically detecting Ac-Lys-H4 and P-Ser-10/Ac-Lys-14-H3 (Cell Signaling, Beverly, MA). In all experiments, ERK2 (Santa Cruz Biotechnologies, Santa Cruz, CA) was detected simultaneously to confirm equal protein loading. Proteins were visualized by incubation with secondary IRDye 800- or Cy5.5-labeled antibodies, respectively (Odyssey infrared imaging system, LI-COR Inc.)

Statistics

Stimulatory effects of *C. pneumoniae* and inhibitory effects were statistically evaluated with an one-way ANOVA with a Dunnett’s post-hoc test. Throughout the figures, $p$ values < 0.05 are indicated by an asterisk.
REFERENCES


