Reduction Display of Tumor Necrosis Factor Receptor I at the Host Cell Surface Supports Infection with Chlamydia trachomatis

The obligate intracellular human pathogenic bacterium Chlamydia trachomatis has evolved multiple mechanisms to circumvent the host immune system. Infected cells exhibit a profound resistance to the induction of apoptosis and down-regulate the expression of major histocompatibility complex class I and class II molecules to evade the cytotoxic effect of effector immune cells. Here we demonstrate the down-regulation of tumor necrosis factor receptor 1 (TNFR1) on the surface of infected cells. Interestingly, other members of the TNF family such as TNFR2 and CD95 (Fas/Apo-1) were not modulated during infection, suggesting a selective mechanism underlying surface reduction of TNFR1. The observed effect was not due to reduced expression since the overall amount of TNFR1 protein was increased in infected cells. TNFR1 accumulated at the chlamydial inclusion and was shed by the infected cell into the culture supernatant. Receptor shedding depended on the infection-induced activation of the MEK-ERK pathway and the metalloproteinase TACE (TNFα-converting enzyme). Our results point to a new function of TNFR1 modulation by C. trachomatis in controlling inflammatory signals during infection.

Chlamydia trachomatis are obligate intracellular bacteria with a unique biphasic developmental cycle. Infectious elementary bodies enter the cell through endocytosis. Inside the cell elementary bodies mature into non-infectious metabolically active reticulate bodies within a vacuole termed the inclusion. At the end of the cycle, reticulate bodies redevelop into elementary bodies that are released from the cell to start a new infection. C. trachomatis is the most common causative agent of sexually transmitted diseases in the western world. In developing countries it is responsible for trachoma, a form of follicular conjunctivitis passed on by smear infection, which is the leading cause of acquired blindness. Chronic infections with C. trachomatis are associated with inflammatory diseases of the joints including reactive or rheumatoid arthritis (1, 2).

The details of how diseases caused by C. trachomatis infection develop are still not fully understood, but immunological responses of the host very likely play a major role (for review, see Ref. 3). These involve the secretion of cytokines and chemokines by infiltrating immune cells. One of the best-studied cytokines is interferon γ produced by activated T-lymphocytes. It limits chlamydial growth by stimulating the synthesis of indoleamine 2,3-dioxygenase, an enzyme that participates in tryptophan catabolism. Therefore, the availability of tryptophan for the bacteria is limited, resulting in reduced growth (4, 5). Tryptophan limitation induced by interferon γ can synergistically be enhanced by tumor necrosis factor α (TNFα) (6) and reversed by supplying additional tryptophan (7). Chlamydial infection activates macrophages to produce TNFα and interferon γ, which is believed to drive an inflammatory loop especially during a persistent infection (8, 9).

However, TNFα also blocks chlamydial growth on its own if applied to host cells before infection (10). TNFα is a cytokine with cytotoxic and antibacterial functions (11) that is produced in high amounts during a chlamydial infection in vitro and in vivo (12, 13). TNFα exerts its pleiotropic effects via two receptors of the tumor necrosis factor receptor superfamily, TNFR1 and TNFR2 (14, 15). Both receptors are involved in the regulation of the immune system and inflammation but via different mechanisms.

TNFR1 contributes to divergent cellular events; that is, survival or apoptosis, depending on the complex formed at the intracellular domain. After binding of TNFα to TNFR1, the so-called complex I comprising TRADD, TRAF2, cIAP-1, cIAP-2, and RIP is formed. This leads to the recruitment of IKK (1×B kinase) followed by activation of NF-κB. After dissociation of complex I from the receptor, complex II composed of FADD (FAS-associated death domain protein) and procaspase-8 can...
be formed. If the activation of NF-κB by complex I was efficient, apoptosis was prevented since cFLIP, a caspase-8 homologue, is expressed, blocking the activation of caspase-8. Apoptosis is only initiated if NF-κB activation was insufficient or was actively inhibited (16). Besides its function in controlling cell survival and apoptosis, NF-κB is a major transcription factor for pro-inflammatory cytokines including interleukin (IL)-8 (17).

TNFR2 contributes to the TNFα-induced effects in two different ways; first, by its own signaling activity, which is a consequence of receptor aggregation, and second, TNF2 controls the access of TNFα to TNFR1 and thereby acts as a decoy receptor for TNFR1. TNFR2 has been shown to bind and sequester TNFα, leading to reduced TNFR1 activation, especially at low TNFα concentrations (18).

TNFR1 and TNFR2 are initially synthesized as membrane-anchored proteins whose extracellular domain can be released by proteolysis, a process termed protein ectodomain shedding (19–23). In the case of TNFR1, this process is mediated by the TNFα-converting enzyme (TACE) (24–26). The proteolytic cleavage of TNFR1 plays an important role in the physiological turnover and regulation of TNFR1 (p55) molecules present on the cell surface. Shedding and the resulting decrease of TNFR1 on the surface transiently desensitizes cells for TNFα action (27). TNFR1 has immunoregulatory functions by inducing apoptosis in monocytes through reverse signaling via transmembrane TNFα (28). Soluble TNFRs are constitutively released into the circulation during various disease states. This activates receptor shedding mechanisms, setting up thresholds for cytokine function to balance resistance and susceptibility to disease (29).

Here we show that C. trachomatis infection differentially affects the surface expression of TNFR1 and TNFR2. We provide a new mechanism of how Chlamydia reduces the bactericidal action of secreted TNFα, a major weapon of the immune system.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures and Chlamydial Growth**—HEp-2 human larynx carcinoma cells (ATCC CCL23), Jurkat T-cells (ATCC TIB-152), and the monocytic cell line U937 (ATCC CRL-2367) were maintained in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated FCS (Invitrogen). The endothelial cell line human umbilical vein endothelial cells (HUVEC; ATCC CRL-1730) was grown in endothelial cell medium enriched with endothelial cell supplement containing 2% FCS (PromoCell). C. trachomatis strain LGV serovar L2 was routinely propagated in HEp-2 cells and purified as previously described (30). Stocks were stored frozen in sucrose-phosphate-glutamate buffer, pH 7.4, at −75 °C and freshly thawed for each experiment.

**Infection with C. trachomatis**—HEp-2 and HUVEC were infected with C. trachomatis at an m.o.i. of 1 or 2 in RPMI 1640 in the presence of 5% FCS and grown at 35 °C and 5% CO2. One hour post-infection the medium was replaced from HUVEC by endothelial cell medium. The suspension cells Jurkat T and U937 were infected by centrifugation at 900 × g at 35 °C for 1 h at an m.o.i. of 10 in RPMI 1640 supplemented with 5% FCS.

**SDS-PAGE and Immuno blotting**—Non-infected and infected HEp-2 cells were grown in six-well plates, washed with PBS, and harvested using a rubber policeman. The cells were pelleted by centrifugation, and the pellets were resuspended in 1× Laemml buffer (3% 2-mercaptoethanol, 20% glycerin, 0.05% bromphenol blue, 3% SDS). After separation by SDS-PAGE, the proteins were transferred to polyvinylidene difluoride membranes (PerkinElmer Life Sciences) and blocked with 3% BSA and 5% milk powder in Tris-buffered saline (TBS) containing 0.5% Tween 20 (TBS-T) for 1 h before incubation with antiserum. The following antisera or antibodies were used: rabbit polyclonal anti-human TNFR1 and rabbit polyclonal anti-human ERK1/2 (Santa Cruz Biotechnology); mouse monoclonal anti-human β-actin or mouse monoclonal anti-human α-tubulin (Sigma Aldrich); mouse monoclonal anti-human phospho-p44/42 ERK1/2 (Thr-202/Tyr-204); rabbit polyclonal anti-human phospho-MEK1/2 (Ser-217/221) or rabbit polyclonal anti-human MEK1/2 (Cell Signaling). Primary bound antibodies were visualized with goat anti-rabbit IgG or rabbit anti-mouse IgG conjugated with horseradish peroxidase (Amersham Biosciences and Jackson ImmunoResearch Laboratories). Band intensity was calculated using the AIDA Image Analyzer Version 4.03 software.

**Indirect Immunofluorescence of Phosphorylated MAPK ERK1/2**—Detection of ERK1/2 by immunofluorescence was performed as previously described (31). Cells were fixed in 2% paraformaldehyde for 30 min at room temperature and permabilized in 1% saponin for 30 min. The primary mouse monoclonal anti-human phospho-p44/42 MAPK ERK1/2 (Thr-202/Tyr-204) antibody (Cell Signaling) was diluted 1:100 in 0.3% saponin and 0.2% BSA and applied to the cells at 4 °C overnight. Unbound antibody was removed by washing 3 times in PBS for 15 min. Secondary Cy3-labeled goat anti-mouse IgG (Jackson ImmunoResearch) was used at a dilution of 1:100. Unbound secondary antibody was removed by three washes in PBS, and coverslips were covered with Moviol.

**Indirect Immunofluorescence of NF-κB (p65) Translocation**—Cells were infected with C. trachomatis at an m.o.i. of 1 or remained uninfected. One day post-infection 50 ng/ml of recombinant human TNFα (Pharmingen) was added. Cells were fixed with acetone/methanol in a 1:2 dilution for 5 min at −20 °C and stained for NF-κB (p65) with a mouse monoclonal anti-human NF-κB (p65) antibody (Santa Cruz Biotechnology) in 3% BSA in PBS and a Cy3-labeled goat anti-mouse IgG (Jackson ImmunoResearch). Samples were viewed under an epifluorescence microscope, Leica DMRB (Leica). Pictures were taken with the Nikon DXM 1200 using ACT-1 software.

**Indirect Immunofluorescence of TNFR1 and Golgi-58K**—Cells were infected with C. trachomatis at an m.o.i. of 1 or remained uninfected. One day post-infection cells were washed with PBS and fixed with 4% paraformaldehyde for 10 min, washed twice in PBS, permeabilized with 1% Triton X-100 for 10 min, and washed in PBS with 0.02% Tween 20 for 5 min before washing in 1% BSA and 0.02% Tween 20 for 5 min. We used a polyclonal rabbit anti-TNFR1 (H-271) (Santa Cruz Biotechnology) and a monoclonal mouse anti-Golgi-58K (Abcam) antibody diluted 1:100 in 3% BSA in PBS and incubated for 45 min at 37 °C. Cells were washed with PBS supplemented with

**Modulation of TNFR1 during Chlamydia Infection**—The expression of TNFR1 was monitored in non-infected HEp-2 cells and the cells infected with C. trachomatis. The expression of TNFR1 was assessed using an antibody against TNFR1. The results showed that the expression of TNFR1 was increased in the infected cells compared to the non-infected cells. This suggests that C. trachomatis infection modulates the expression of TNFR1.
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1% BSA and 0.02% Tween 20, and a donkey anti-rabbit-Cy3 or rabbit anti-mouse-Cy3 (Jackson ImmunoResearch) was added for 45 min at room temperature. Chlamydial inclusions and nuclei were stained by adding 5 μM DNA dye DRAQ5 to the secondary antibody solution. Unbound antibodies were removed by washing in 0.02% Tween 20 for 5 min followed by a final washing step in PBS. Samples were viewed under a confocal microscope TCS SP-1 (Leica Instruments) at 630-fold magnification.

Flow Cytometry—For surface staining of receptors, cells were grown in 24-well plates and detached by Accutase (Promocell) treatment at 35 °C. Cells were washed 3 times in PBS containing 0.05% BSA (blocking buffer) by centrifugation at 300 × g for 5 min. Antibodies directed against TNFRs (monoclonal anti-human TNFR1/TNFRSF1A-phycocerythrin, IgG1, monoclonal mouse anti-human TNFR2-phycocerythrin, IgG2A; R&D Systems), CD95 (mouse monoclonal anti-human CD95-fluorescein isothiocyanate (FITC), Immunochemistry Systems), or TRAIL-R2 (mouse monoclonal anti-human FITC-TRAIL-R2, HS201, Alexis Biochemicals) and isotype control immunoglobulins (R-(PE)- and R-(FITC)-conjugated mouse IgG1,κ or R-(PE) conjugated mouse IgG2A,κ (Pharmingen) were diluted 1:3 in blocking solution and applied to cells for 25 min at room temperature in the dark followed by a washing step in blocking buffer. Cells were then fixed in 0.05% formalin and measured in a FACSCalibur (BD Biosciences) using an excitation wavelength of 488 nm and emission filters of 525 and 575 nm, respectively.

FACS analysis at 525 nm using an excitation wavelength of 488 nm and emission filters of 525 and 575 nm, respectively.

Transfection of siRNAs and Subsequent Infection—HEp-2 cells were grown in 6-well plates 24 h before siRNA transfection. Transfection of siRNAs against TNFR1, TNFR2, or a control siRNA against luciferase was carried out with the RNAiFect kit (Qiagen). One day post-transfection cells were trypsinized and transferred to 12-well plates for FACS analysis, to 6-well plates for immunoblotting, or to 48-well plates for TNFα challenging experiments. The cells were then infected with C. trachomatis 48 h post-transfection, and 24 h later the specified experiments were performed. The targetting sequences of the employed siRNAs were as follows: siLuciferase, 5′-AACUUA- CCGUGAGACUUCUGA-3′; siTNFR1, 5′-GGGCACAAA-GGAAACCUACDdTdT-3′; siTNFR2, 5′-AAGAAACCAGC- AUCUGCAACC.

Quantification of mRNAs by Real-time RT-PCR—RNA was isolated from infected HEp-2 cells by using Trizol® reagent (Invitrogen) according to the manufacturer’s instructions. The relative amount of specified mRNA was determined by real-time PCR using a Quantitect™ SYBR® Green RT-PCR kit (Qia- gen) following the manufacturer’s instructions. The expression level of mRNA was normalized against the internal standard GAPDH. The following primers were used: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-GGTATCTGGAGAA-GGACTCATGAC and 3′-ATGCCAGTGAAGCTCTCCGT- CAG; TNFR1, 5′-ACAGGGAGAAGAGAGATATG and 3′- AAGAGAGAGACTTCCACCTGAC; TNFR2, 5′-GACTCTT- GTGACAGAGCAGG and 3′-TGTTTCCGATTCGTCGTTG.

Infectivity Assays and Automated Microscopy—HEp-2 cells were transfected or treated as described before infection. Two days post-infection, cells were lysed with 0.06% Nonidet P-40 for 15 min at room temperature, and lysates were diluted 1:100 in RPMI 1640 with 5% FCS before transfer to fresh untreated HEp-2 cells in 48-well plates. After growth for 24 h at 35 °C and 5% CO2, the cells were fixed in methanol for 10 min, washed in PBS, and blocked in 0.2% BSA in PBS for 30 min at room temperature. Primary mouse anti-C. trachomatis-MOMP KK12 (University of Washington) was added at 1:10,000 in blocking solution for 1 h at room temperature and Cy3-labeled goat anti-mouse IgG (Jackson ImmunoResearch) at 1:100 in blocking solution for another hour. Host cell nuclei were stained with Hoechst 33342 (Merek) diluted 1:1000 in blocking solution of the secondary antibody. Chlamydial inclusion size and number of cells and Chlamydia were analyzed in an automated microscope (Olympus Biosystems). Four pictures were taken from each well with a UV filter and a Cy3 filter at the same position. The images were analyzed for the number of cells by automatically identifying and counting the host cell nuclei, and the number and size of chlamydial inclusions were determined using ScanR Analysis (Olympus Biosystems).

Statistics—For statistical analysis and graphs, Microsoft Excel 2002 for Windows XP Home was used. For determination of the statistic significance, Student’s test was performed. p values <0.05 were considered as statistically significant.

RESULTS

The Surface Expression of TNFR1 Was Reduced in HEp-2 Cells during Infection with C. trachomatis—To investigate the fate of TNFR1 during infection, HEp-2 cells were infected with...
C. trachomatis for 24 h and stained with a monoclonal TNFR1 antibody. FACS analysis revealed reduced amounts of TNFR1 on the surface of infected cells compared with non-infected cells (Fig. 1A). TNFR1 was not reduced on cells infected with UV-inactivated bacteria (Fig. 1A). For a more detailed investigation of the reduction of TNFR1, a time course experiment was performed. HEp-2 cells were mock-infected or infected with C. trachomatis for 2, 4, 10, 15, 24, or 30 h and stained with TNFR1 antibody. FACS analysis revealed reduced amounts of TNFR1 on the surface of infected cells compared with non-infected cells (Fig. 1B). TNFR1 was not reduced on cells infected with UV-inactivated C. trachomatis for 2, 4, 10, 15, 24, or 30 h.
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UV-inactivated *C. trachomatis* for 10 and 24 h and stained under the same conditions as above for FACS analysis. Reduction of TNFR1 from the surface to 76.9% compared with mock-infected cells was already observed 10 h post-infection, which increased to 57.2% (p ≤ 0.001) at 15 h and to 38.3% (p ≤ 0.0001) 24 h post-infection. Infection with UV-inactivated *Chlamydia* only insignificantly reduced TNFR1 at 24 h post-infection to 89.4% (p ≥ 0.1) (Fig. 1B), suggesting that the reduction of TNFR1 was dependent on growing, metabolically active bacteria.

To test whether the reduction of TNFR1 was cell type-specific, monocytic-like U937, HUVEC, and Jurkat T-cells were infected with *C. trachomatis* for 24 h, and surface exposure of TNFR1 was measured by FACS analysis. TNFR1 was present in reduced amounts on all tested cell lines (Fig. 1, C and D), demonstrating a cell line-independent effect of infection on TNFR1 surface display.

TNFR1 belongs to the TNFα receptor superfamily. Receptors of this family contain an intracellular domain with a death domain, which is responsible for the transduction of apoptotic signals after ligand binding. We tested other death receptors, TRAIL-R2 and CD95 (Fas/Apo1), to correlate the presence of the death domain with receptor down-regulation by infection. HUVEC were infected at an m.o.i. of 2 and Jurkat T-cells at an m.o.i. of 10, and surface exposure of TRAIL-R2 and CD95 (Fas/Apo1) was measured 24 h later. The amount of TRAIL (DR5) on the surface of infected HUVEC decreased to 31.5% compared with mock-infected cells, whereas the amount of CD95 (Fas/Apo1) remained unaffected (Fig. 1E). Next, the regulation of TNFR2, another receptor belonging to the TNFα receptor superfamily but lacking a death domain, was investigated as before. TNFR2 was not regulated during an infection with *C. trachomatis* (Fig. 1E). Activation of TNF receptors leads to the activation of NF-κB and transcription of IL-8, which binds to IL-8 receptors after secretion. We, therefore, tested the fate of IL-8 receptor, which was, however, not affected by infection (Fig. 1E, lower panels). Thus, the observed effect is not specific for TNFR1, but not all death domain-containing receptors are reduced from the surface of infected cells.

*Expression of TNFR1 and TNFR2 Is Up-regulated in Infected Cells*—An obvious reason for the observed reduction of TNFR1 could be the reduced expression in infected cells. We, therefore, measured the amount of TNFR1 and TNFR2 mRNA from control and infected cells by quantitative real-time RT-PCR. Surprisingly, TNFR1 mRNA was 3-fold, and that of TNFR2 was 5-fold up-regulated in infected cells (Fig. 2A). To test whether increased mRNA correlated with higher protein concentrations, TNFR1 was analyzed in permeabilized cells by FACS analysis. The intracellular amount of TNFR1 protein was 1.4-fold increased in infected cells compared with the control 24 h post-infection (Fig. 2, B and C). In addition, immunoblot analysis was performed to further confirm these results. The level of TNFR1 was increased in infected cells compared with non-infected cells or cells infected with UV-inactivated *C. trachomatis* (Fig. 2D), demonstrating an increase in TNFR1 upon infection. Moreover, an additional TNFR1 band appeared in the immunoblots of infected cells (Fig. 2D), suggesting a modification of the receptor in infected cells.

**FIGURE 2. Expression of TNFR1 and TNFR2 is increased in infected cells.** A, increased mRNA levels of TNFR1 and TNFR2 in infected cells. HEp-2 cells were either mock-infected or infected with *C. trachomatis*, and the relative (rel.) amount of TNFR1 or TNFR2 mRNA was analyzed by quantitative real-time RT-PCR. The mean mRNA level of TNFR1 increased 3-fold; that of TNFR2 5-fold upon infection. Error bars show the S.D. of three independent experiments. B–D, level of TNFR1 is elevated in infected cells. FACS analysis of intracellular TNFR1 (B) and subsequent quantification (C) revealed an up-regulation of TNFR1 24 h post-infection. In B, the black line represents the isotype control from mock-infected cells, the dotted line represents the isotype control from infected cells, the light gray-filled graph indicates TNFR1 in mock-infected cells, and the dark gray-filled graph shows TNFR1 from infected cells. Error bars in C represent the S.D. of three independent experiments (⁎, p ≤ 0.05). D, consistently, immunoblot analysis showed a strong increase in the amount of TNFR1 protein, and an additional band appeared above the main TNFR1 band (indicated with an arrow) in infected cells compared with mock-infected cells and cells infected with UV-inactivated bacteria.

Surface Display of TNFR1 Is Controlled by MAPK Pathways in Infected Cells—Reduction of TNFR1 protein from the surface has been shown before to be controlled by ERK1/2 (32). Because *Chlamydia* infection activates MAPKs in other cells (31), we reasoned that this pathway may be involved in the

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We then established the inhibition of MEK1 with the specific inhibitor U0126 to test whether MAPK activation is required for the reduction of TNFR1 from the surface. The inhibitor was added to the cells at 10, 25, and 50 μM, and the cells were infected with C. trachomatis with an m.o.i. of 1. U0126 was well tolerated by the cells at all concentrations. The inclusion size was reduced at 50 but not at 10 or 25 μM U0126 (not shown). Therefore, only concentrations of 10 or 25 μM U0126 were used for further analysis. U0126 strongly blocked Chlamydia-induced ERK1/2 phosphorylation at 25 μM but was inefficient at 10 μM. The infection-induced up-regulation of TNFR1 protein was prevented in cells treated with 25 μM U0126 (Fig. 3, C and D). To check whether surface exposition of TNFR1 was affected by MAPK inhibition, U0126-treated and -infected cells were analyzed by FACS. The MEK1 inhibitor caused a general concentration-dependent increase of TNFR1 on the surface, but infected treated cells had significantly more TNFR1 on the surface compared with non-treated infected cells (Fig. 3E). Whereas infection caused a reduction of TNFR1 surface display by 60.1% in non-treated cells, the reduction in response to infection was only 43.2 and 34.5% in cells treated with 10 and 25 μM U0126, respectively (Fig. 3F). These results suggested that the reduction of TNFR1 from the surface may involve the activation of ERK1/2.

**Golgi-dependent Translocation of TNFR1 to the Chlamydial Inclusions**—The ERK1/2-dependent reduction of TNFR1 has been linked to the sequestration of receptor in the endoplasmic reticulum (ER) (32), which may explain why in the infected cells the overall TNFR1 concentration increased, whereas surface-exposed TNFR1 decreased. We, therefore, verified whether TNFR1 localizes to different cellular compartments in the infected and non-infected cells. Microscopic analyses of cells transfected with ER-targeted DsRED revealed no increased co-localization of TNFR1 to the ER in the infected cells (data not shown). However, TNFR1 localized to the chlamydial inclusions and Golgi

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**FIGURE 3. Reduction of surface TNFR1 on infected HEp-2 cells is controlled by the MEK-ERK pathway.** A, activation of the MEK-ERK pathway correlates with the modification of TNFR1. HEp-2 cells were either mock-infected or infected with C. trachomatis for the indicated time points and subsequently subjected to immunoblot analysis for phosphorylated (p) ERK1/2p42/44, phosphorylated MEK1/2, and MEK1/2. The increase in ERK1/2p42/44 and MEK1/2 phosphorylation correlated with an increase in the overall amount of TNFR1 protein and the appearance of an additional band, the TNFR1 band indicated with arrows. B, infected cells contain high amounts of phosphorylated ERK1/2p42/44 (red). Cells were stained with an antibody against phosphorylated ERK1/2p42/44 24 h post-infection and analyzed by confocal laser scanning microscopy. Cells containing chlamydial inclusions (marked with an arrow) also have phosphorylated ERK1/2p42/44. C–F, HEp-2 cells were infected for 24 h and treated with U0126 as indicated. Inhibition of ERK activation decreased infection-induced TNFR1 levels and modification (C). Quantification of the band intensity from the Western blot, presented in C. Band intensity was calculated using the AIDA Image Analyzer Version 4.03 software (D). U0126 reversed the infection-induced reduction of surface TNFR1. TNFR1 was analyzed in Me2SO-treated control cells, cells treated with 10 μM U0126, and 25 μM U0126 by FACS. The black line shows the isotype control from mock-infected cells, the dotted line shows the isotype control from infected cells, the light gray-filled graph shows the level of the receptor on the surface of mock-infected cells, and the dark gray-filled area shows the amount of the receptors on the surface of infected cells (E). Quantification of the experiment is shown in F. Error bars represent ± S.D. of three independent experiments.

reduction of TNFR1 surface display in HEp-2 cells. HEp-2 cells were infected with C. trachomatis for 4, 10, 15, 18, or 24 h, and activation of ERK1/2 and of the ERK kinase MEK1 was tested by immunoblot analysis with phospho-specific antisera. Both MEK1/2 and ERK1/2 were activated in infected cells in a time frame of 15–24 h (Fig. 3A). The phosphorylated form of ERK1/2 co-localized to the cells carrying inclusions (Fig. 3B), suggesting a direct activation of ERK1/2 by Chlamydia in HEp-2 cells.
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apparatus, as was shown by co-staining of the Golgi formiminotransferase cyclodeaminase (Golgi-58K) with the chlamydial inclusion (Fig. 4A). Interestingly, blocking MAPK signaling by U0126 treatment had no effect on the recruitment of TNFR1 to the chlamydial inclusion (data not shown). To analyze whether transport of TNFR1 to the inclusion depends on an intact Golgi apparatus, cells were preincubated with brefeldin A, a substance that blocks the protein transport from ER to the Golgi complex and induces accumulation of proteins in the endoplasmic reticulum. The addition of brefeldin A for 24 h led to the reduction of TNFR1 on the surface in uninfected cells similar to the reduction in the non-treated but infected cells (Fig. 4C). Under these conditions, the colocalization of TNFR1 with the chlamydial inclusions was no longer observed (Fig. 4B), suggesting that TNFR1 was transported to the inclusions in a Golgi-dependent manner.

Formation of sTNFR1 by Infected Cells—Another well known mechanism, by which TNFR1 is reduced from the cell surface, is the shedding of the extracellular domain into the cell surroundings forming a soluble receptor (sTNFR1). We, therefore, tested whether infected cells release sTNFR1 into the culture supernatant. The supernatants of infected HeP-2 cells were collected at 6, 16, 24, and 48 h post-infection and tested for soluble receptor by ELISA. The amount of sTNFR1 strongly increased in a time-dependent manner, with 41 pg/ml sTNFR1 16 h, 436 pg/ml 24 h, and 21.5 ng/ml 48 h post-infection. Interestingly, the MEK1 inhibitor U0126 drastically reduced the amount of sTNFR1 to the basal level of uninfected cells (Fig. 4D), suggesting an involvement of MAPKs in the infection-induced shedding of TNFR1.

Shedding of TNFR1 is mediated by TACE (ADAM17) (24, 25), a metalloproteinase localized to the cytoplasmic membrane. We, therefore, tested if TAPI-1, an inhibitor of TACE, could block TNFR1 release into the supernatant. Because previous reports demonstrated a bactericidal activity of metalloprotease inhibitors on Chlamydia (33), the toxicity of TAPI-1 was carefully investigated. The addition of TAPI-1 up to 10 μM did not influence chlamydial growth (data not shown). Interestingly, the addition of as little as 1 μM TAPI-1 reduced the concentration of sTNFR1 in the supernatant from the infected cells (Fig. 4E). TAPI-1 added at a concentration of 10 μM completely blocked the infection-induced release of the ectodomain of TNFR1 (Fig. 4E) but also caused a reduction of surface-exposed TNFR1 (Fig. 4F). Because infected TAPI-1-treated cells still displayed significant levels of TNFR1 at their surface (Fig. 4F) but failed to secrete sTNFR1 into the supernatant (Fig. 4E), we concluded that TACE is the protease involved in the shedding of TNFR1 in infected cells.

Signal Transduction through TNFRs Is Affected in Infected Cells—TNFR1 and TNFR2 bind soluble and membrane-bound TNF, respectively. We, therefore, investigated if the surface reduction impaired TNF-induced signal transduction via TNFR. A well documented consequence of TNFR activation is the translocation of NF-κB (p65) from the cytosol to the nucleus. Therefore, HeP-2 cells were infected and treated with TNFα at different time points to check NF-κB (p65) translocation. A complete translocation of NF-κB (p65) could be observed in mock-infected cells after 20 min but only in 40% of the infected cells. 75 min after the addition of TNFα, 60% of NF-κB (p65) had relocated to the cytoplasm in mock-infected cells, whereas in infected cells a peak of p65 translocation of 80% was observed in infected cells (Fig. 5, A and B), demonstrating a delay in NF-κB translocation.

NF-κB regulates the transcription of a number of diverse genes including IL-8. To test whether the delay in NF-κB (p65) translocation had any effect on the transcription of the IL-8 gene and its subsequent secretion, IL-8 was measured in response to TNFα treatment and the blockade of MAPK signaling by U0126 treatment in the supernatant of infected cells. Infection triggered the secretion of IL-8, which was strongly reduced by! the MEK1 inhibitor U0126 (Fig. 5C). The addition of TNFα led to the secretion of IL-8 in a time-dependent manner from mock-infected cells or infected cells. In general, however, more IL-8 was secreted from infected cells compared with non-infected cells, suggesting that the reduction from the surface has no obvious effect on late signaling events transmitted by TNFR1.

Control of C. trachomatis Infectivity by TNFR1—Because only TNFR1, but not TNFR2, was reduced on the surface of infected cells but long term signal transduction via TNFR was not impaired, we asked whether down-regulation of TNFR1 has any advantage for chlamydial growth. The expression of TNFR1 and TNFR2 was silenced by RNA interference in infected cells, and the effect on the chlamydial infectivity was tested. Silencing of TNFR1 or TNFR2 by RNA interference had no influence on the primary infection (data not shown). To test for infectivity, Chlamydia were passaged onto fresh HeP-2 cells 2 days post-infection, and developing inclusions were counted by automated microscopy. Chlamydia from SiLUCiferase-transfected cells had about 900,000 infection-forming units/ml and were used for standardization. Silencing of TNFR1 or TNFR2 alone had no influence on the infectivity of C. trachomatis (Fig. 6, A and B). However, when infected cells were challenged with TNFα, infectivity increased to 112% that of control in TNFR1 knockdown cells but dropped to 50% in SiLUCiferase and to 53% in TNFR2 knockdown cells (Fig. 6, A and B). The MEK1 inhibitor U0126 used at 25 μM dramatically reduced the infectivity of C. trachomatis under all conditions to an average of 30% control (Fig. 6, A and B). In conclusion, reduction of TNFR1, but not TNFR2, is advantageous for chlamydial infectivity when TNFα concentrations are high.

DISCUSSION

Obligate intracellular human pathogenic bacteria like C. trachomatis are constantly exposed to multiple powerful bactericidal activities of the immune system. Therefore, bacteria in return develop strategies to circumvent, counteract, or augment the host immune response while establishing an acute infection or long term persistency. For example, Chlamydia actively prevent host cell apoptosis during acute (34–36) and persistent infection (37) or directly interfere with the immune response, i.e., by down-regulation of the expression of major histocompatibility complex class I and class II molecules (38–40). Here, we demonstrate a down-regulation of TNFR1 on the surface of infected cells. TNFR1 surface down-regulation was achieved by its recruitment to chlamydial inclusions and shed-
FIGURE 4. TNFR1 colocalizes with the inclusion membrane and the Golgi apparatus. A, HEp-2 cells were infected with C. trachomatis or mock-infected for 24 h. Cells were fixed and stained to visualize Golgi-58K (green) and TNFR1 (red). Chlamydia were visualized using the DNA dye DRAQ5 (blue, see arrowheads). Colocalization of Golgi with TNFR1 appears yellow in the overlay; that of TNFR1 with chlamydial inclusions appears purple. B, HEp-2 cells were infected with C. trachomatis or mock-infected for 24 h and treated with 2.5 μM brefeldin A at the time of infection. Cells were fixed and stained to visualize Golgi-58K (red) and TNFR1 (green). Chlamydia were visualized using the DNA dye DRAQ5 (blue, see arrowheads). C, Golgi disruption causes down-regulation of surface-exposed TNFR1. Brefeldin A causes the reduction of TNFR1 from the surface in uninfected cells similar to the reduction in infected cells. HEp-2 cells were infected with C. trachomatis (Ctr) or mock-infected. Mock-infected cells were additionally treated with 2.5 μM brefeldin A (BreA) as indicated. The distribution of TNFR1 on the surface was measured by FACS analysis. The mean fluorescence values for infected cells were calculated relative to mock-infected HEp-2 cells (100%). Error bars represent the mean ± S.D. of three independent experiments (**, p < 0.001). D, release of sTNFR1 by C. trachomatis infected cells. HEp-2 cells were infected with C. trachomatis or mock-infected for 6, 16, 24, and 48 h and treated with 25 μM U0126 as indicated. The supernatant was tested for the presence of soluble TNFR1 by ELISA. Error bars represent the S.D. of two different measurements (p < 0.05). The graph is representative of three different biological replicates. E, release of sTNFR1 by C. trachomatis is inhibited by the metalloprotease inhibitor TAPI-1. HEp-2 cells were infected with C. trachomatis (Ctr) or mock-infected and treated with 1 or 10 μM TAPI-1 for 24 h as indicated, and the presence of sTNFR1 was tested in the supernatant. Error bars represent the S.D. of three independent experiments. F, reduction of surface-exposed TNFR1 by TACE inhibition. Cells either left untreated (-) or infected with C. trachomatis (Ctr) or treated with 10 μM TAPI-1 or infected and treated with both were analyzed by FACS for surface-exposed TNFR1. Shown are the results from one representative experiment of three.
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Figure 5. Delayed signal transduction through TNFR1. A, delayed translocation of TNFα-induced NF-κB (p65) in infected cells. HEP-2 cells were infected with C. trachomatis (bottom panel) or mock (top panel)-infected for 24 h. TNFα was added, and cells were fixed at different time points and stained for NF-κB (p65). Nuclear translocation of NF-κB (p65) (red staining) is delayed in infected cells (green staining) compared with non-infected cells. B, time course of NF-κB (p65) translocation induced by 50 μg/ml TNFα in infected and non-infected cells. C, TNFα-induced IL-8 secretion is not affected in infected cells. HEP-2 cells were infected with C. trachomatis or mock-infected and 24 h post-infection treated with 40 ng/ml TNFα or 25 μM U0126. The release of IL-8 was measured by ELISA 8 or 24 h after the addition of TNFα and/or U0126. Error bars represent the ±S.D. of three independent experiments.

Figure 6. Depletion of TNFR1 or increasing sTNFR1 protects C. trachomatis from bactericidal TNFα effects. A, MAPK inhibition reduces and TNFR1 knockdown increases C. trachomatis infectivity. Chlamydia were grown in HEP-2 cells transfected with a siRNA against Luciferase, TNFR1 or TNFR2, or treated with U0126 (25 μM) or TNFα (25 ng/ml) as described under “Experimental Procedures.” The bacteria were then transferred to fresh, non-treated HEP-2 cells, and the infectivity was analyzed by immunofluorescence staining of chlamydial inclusions. Shown are immunofluorescence pictures of Hoechst stained HEP-2 cells (blue) and chlamydial inclusions (red). B, quantification of the experiments shown in A. The number of inclusions was determined by automated microscopy and expressed as a percentage of siLuciferase-transfected, -infected cells. Shown is the result of one representative of five independent experiments.

ding to the supernatant. We provide evidence for a new strategy used by C. trachomatis to protect the host cell from bactericidal action of TNFα.

The process of reduction of surface exposed TNFR1 is dependent on growing metabolically active bacteria. Moreover, similar effects were observed with diverse cell types such as epithelial and endothelial cells, monocyctic cells, and T cells. We, therefore, assume a general mechanism underlying TNFR1 reduction by C. trachomatis that may not depend on well known inducers of pathogen-associated molecular pattern receptors like lipopolysaccharide. Therefore, a chlamydial protein like the previously described Chlamydia protein associating with death domain (CADD) is a possible candidate. CADD is localized in the inclusion membrane and interacts with the death receptors of CD95, TNFR1, TRAIL-R4 (DR4), and TRAIL-R2 (DR5) at late time points of infection (41), suggesting a mechanism of receptor sequestration by the bacteria. However, we can exclude a mechanism solely involving the death domain since surface-exposed CD95(Fas/Apo1) was not reduced upon C. trachomatis infection. An involvement of chlamydial CADD in the observed surface depletion of receptor from infected cells is, therefore, unlikely.

Overall production of TNFR1 surprisingly increased in response to chlamydial infection, ruling out a reduced TNFR1 expression as a reason for its reduced surface exposure. These findings are in agreement with a recent report on increased TNFR1 levels in cells infected with Chlamydothila psittaci (42). One could assume a feedback regulation driven by the reduced presence of TNFR1 at the surface of infected cells.

Localization studies have shown a redistribution of TNFR1 to the inclusions and the trans-Golgi network, an effect that was abolished by the Golgi-disrupting drug brefeldin A. Membrane receptors like TNFR1 are either located in the trans-Golgi network inside the cell (43) or signal in association with lipid rafts (44, 45). It is interesting to note that lipid rafts are rich in cholesterol and sphingolipids (46, 47), both of which are also present in high amounts in the chlamydial inclusion membrane (48, 49). It is, therefore, possible that TNFR1 translocates from the trans-Golgi network to the chlamydial inclusions during recruitment of cholesterol and sphingolipids to the inclusion membrane. Another possibility is that a TNFR1 pool localizes
in the Golgi to serve as a reservoir for sTNFR1, reducing cell responses to TNF (50).

The increased expression of TNFR1 was controlled by the MEK-ERK pathway since inhibition of MEK1/2 led to a decrease in the concentration of TNFR1. This pathway, therefore, plays a major role in controlling TNFR1 levels during infection, since chlamydial infections were known to activate the ERK pathway (31, 51). We found an additional TNFR1 band in the immunoblot from infected cells, suggesting a modification of the receptor. This modification could be phosphorylation since TNFR1 has been shown to be a direct substrate for the MAPK ERK1/2 (24, 25) affecting the subcellular redistribution of the receptor from the Golgi to tubular structures associated with the ER (32). This possibility could be ruled out because TNFR1 was not found in the ER but in the trans-Golgi network.

The cells infected with C. trachomatis released significant amounts of TNFR1 receptor into the culture supernatant. TNFR1 is synthesized as a membrane protein with an extracellular domain that can be released after TNFα stimulation from the surface by shedding (19, 22, 23). The release of the extracellular domain of TNFR1 and the resulting decrease of the number of receptor molecules on the surface led to the desensitization of the cell for the TNFα effects (27). Lipopolysaccharide could be a possible trigger for the release of sTNFR, as has recently been demonstrated. Enterobacterial lipopolysaccharide causes the production of sTNFR2 and not TNFR1 via a protein kinase C- and Src-independent pathway mediated by p38 MAPK, furin, and metalloproteinases and independent of MEK1/2 (52). However, although a role of chlamydial lipopolysaccharide in the shedding of TNFR1 cannot be excluded, it is unlikely because killed bacteria did not reduce TNFR1 from the surface of cells.

Shedding of TNFR1 is catalyzed by the action of matrix metalloproteinase TACE (24, 25) among other enzymes. We demonstrated that shedding of TNFR1 from the infected cells can be blocked by TAPI-1, an inhibitor of TACE in a concentration-dependent way. Furthermore, reduction of TNFR1 from the cell surface and shedding of TNFR1 was mediated by the activation of the MEK-ERK pathway. Recently, it was demonstrated that TACE is phosphorylated by ERK but not by p38 or JNK (53). Phosphorylation has a potential role in TACE protein trafficking to the cell membrane (54). Consistently, Chlamydia-triggered release of sTNFR1 was dependent on MEK1/2 activity, whereas blocking p38 MAPK by inhibitors or JNK by siRNA was not (data not shown), suggesting that the above-described mechanisms may account for the shedding of TNFR1. Because TACE is included in lipid rafts during its transport through the Golgi (55), it is possible that TACE similarly to TNFR1 is recruited to the chlamydial inclusions in association with cholesterol and sphingolipids. TNFR1 shedding by infected cells may either augment its signal transduction capacity or lead to sequestration of bactericidal TNFα in the vicinity of the cell. The results in our in vitro experiments favor a role of TNFR1 modulation in short term rather than long term signaling since translocation of NR-κB in infected cells was clearly delayed after stimulation with TNFα, whereas TNF-induced IL-8 secretion, which depends on NF-κB, even increased in the infected cell. It is very likely that increased IL-8 secretion is triggered by multiple pathways in the infected cells; one also involving the MEK-ERK activation as MEK inhibition reduced the secretion of IL-8 in infected cells (Fig. 5C). In addition to signaling, we obtained clear evidence for a role in the modulation of TNFR1 in preventing anti-chlamydial activities. Knockdown of TNFR1, but not TNFR2, had a strong effect on chlamydial growth in cells challenged with TNFα. It was also shown recently that TNFR1 but not TNFR2 had significant impact on the inflammatory response (56). This result supported the general role of TNFR1 in the anti-chlamydial host response.

The regulation of TNFR1 is also found with other pathogens, mainly viruses. The Epstein-Barr virus immediate early protein BZLF1 down-regulates TNFR1 expression and thereby prevents TNFα-mediated apoptosis, circumventing antiviral TNFα effects (57). Another example is the human cytomegalovirus, subverting host antiviral activities by reducing TNFR1 from the cell surface in the course of infection (58). Shigella spp. selectively down-regulate surface receptors, which can bind anti-bacterial cytokines, including the TNFR1 (59). Thus, our finding of a TNFα-blocking activity induced by C. trachomatis fits well to the already recognized strategy used by pathogens to subvert the action of host anti-microbial cytokines. Down-regulation of TNFR1 from the surface of infected cells by shedding into the supernatant and recruitment to the chlamydial inclusions is a new mechanism that may allow the pathogen to suppress the immune system and to establish a stable infection.

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REFERENCES

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