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Increased inflammation and impaired resistance to *Chlamydophila pneumoniae* infection in *Dusp1*$^{-/-}$ mice: critical role of IL-6

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ABSTRACT

The MAPK phosphatase DUSP1 is an essential negative regulator of TLR-triggered innate immune activation. Here, we have investigated the impact of DUSP1 on inflammatory and antimicrobial host responses to the intracellular pathogen *Chlamydophila pneumoniae*. Following nasal infection, DUSP1-deficient mice mounted an enhanced pulmonary cytokine (IL-1β, IL-6) and chemokine response (CCL3, CCL4, CXCL1, CXCL2), leading to increased leukocyte infiltration. Of interest, the increased inflammatory response, in the absence of DUSP1, was associated with higher bacterial numbers in the lungs, although the expression of IFN-γ and critical antichlamydial effector molecules, such as iNOS, was intact. Blockade of IL-6 trans-signaling by injection of a soluble gp130-Fc fusion protein corrected the overshooting chemokine production as well as the increased chlamydial load in *Dusp1*$^{-/-}$ mice. Furthermore, IL-6 enhanced the replication of *C. pneumoniae* in embryonic fibroblasts in vitro. These data show that DUSP1 is required to achieve a balanced response to chlamydial infection and identify IL-6 as critical for amplifying inflammation and benefiting chlamydial growth through direct effects on infected cells. *J. Leukoc. Biol.* 88: 579–587; 2010.

Introduction

Cytokine and chemokine production in response to infectious danger is triggered through the activation of MAPK and NF-κB signal transduction pathways downstream of pattern recognition receptors. This initial response leads to recruitment of leukocytes that are required to fend off infection. In addition, the inflammatory signals drive the full expression of antimicrobial effector programs, such as production of reactive oxygen species and nitrogen intermediates. A rapid, innate response is therefore a prerequisite for control of microbial replication. However, it also carries the risk of collateral damage to the host tissues, creating a necessity for endogenous negative regulators that act at many different levels. It is often assumed that these molecular brakes impair the development and execution of optimal host antimicrobial responses.

The MAPK signaling module is controlled by a family of DUSP that binds to activated MAPK and terminates their activity by dephosphorylation of critical tyrosine and threonine residues [1]. Recently, at least three family members have been shown to be functionally relevant in the control of immune responses [2]. DUSP10/MKP-5 primarily targets JNK and affects innate and adaptive responses [3]. DUSP2/phosphatase of activated cell-1 is induced by receptor ligation in many innate and adaptive immune cells and is involved in cross-talk regulation of ERK1/2 by JNK [4]. DUSP1/MKP-1, the founding member of the DUSP family, targets mainly p38 MAPK and is essential for restricting the response of macrophages and dendritic cells to TLR ligands [5–8]. The unrestricted output of a subset of LPS-induced cytokines (e.g., TNF-α, IL-6) and chemokines (e.g., CCL3, CCL4) in *Dusp1*$^{-/-}$ mice correlates with the strongly increased lethality in the high-dose endotoxin shock model.

The question of whether during infection with live microbes, a stronger activation of the p38 MAPK pathway in the absence of DUSP1-mediated control causes detrimental immunopathology or may lead to a beneficial increase in resistance has been addressed recently for infection with the gram-positive bacterium *Staphylococcus aureus* [9]. In this setting, *Dusp1*$^{-/-}$ mice again showed overproduction of some cyto-

Abbreviations: APC=allophycocyanin, DDCT=$\Delta\Delta$ comparative threshold, DUSP1=dual-specificity phosphatase 1, IFU=inclusion-forming unit(s), iNOS=inducible NO synthase, MEF=murine embryonic fibroblast, MKP=MAPK phosphatase, MOI=multiplicity of infection, MPO=myeloperoxidase, qRT-PCR=quantitative RT-PCR, slL-6Ra=soluble IL-6Rα chain, SOCS=suppressor of cytokine signaling, WT=wild-type

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kines and chemokines. *Dusp1*$^{-/-}$ mice succumbed to injection of doses of heat-killed *S. aureus* that were nonlethal in WT controls. Of interest, however, the increased inflammatory response in the absence of DUSP1 did not affect bacterial replication nor did it affect the survival after infection with live *S. aureus* [9].

Chlamydophila pneumoniae is an intracellular pathogen that infects the respiratory tract in humans [10] and mice [11]. Respiratory epithelial cells are infected by elementary bodies that survive and replicate inside an inclusion by developing into reticular bodies that divide by binary fission. After 2–3 days, the infected cell releases a multitude of elementary bodies that infect neighboring cells [11]. Immune control of chlamydial infection relies mainly on IFN-γ-mediated responses [12], which involve, among others, the action of iNOS [13] and several IFN-induced 47 kDa GTPases [14, 15]. The innate immune system senses *C. pneumoniae* via TLR2 and TLR4, and the associated adaptor protein MyD88 plays a critical role in this process. Although MyD88-deficient as well as TLR2$^{-/-}$ mice are unable to control the infection and die after 8–10 days with high titers of chlamydial burden in the lungs, it is of interest that the mitigated inflammatory response with a low number of pulmonary granulocytes during the early stage of infection is associated with a reduced number of *C. pneumoniae* in the lung at Day 3 postinfection [16–18]. Not surprisingly, the MAPK pathway is activated by *C. pneumoniae* infection in macrophages and epithelial cells in vitro and contributes to the production of cytokines such as GM-CSF [19] and mediators such as NO [20]. However, there are no data about the role of MAPK activation in the orchestration of inflammatory responses to *C. pneumoniae* infection in vivo.

Here, we have investigated the phenotype of *Dusp1*$^{-/-}$ mice in pulmonary infection with *C. pneumoniae*. We found an increased inflammatory response in the lungs, which was, however, not effective in boosting resistance but rather, promotes the pathogen through IL-6 trans-signaling.

MATERIALS AND METHODS

Mice

Dusp1$^{-/-}$ mice, back-crossed for eight generations onto a C3H background, were obtained initially from Dr. Andrew Cato (Forschungszentrum Karlsruhe, Germany) with permission from Bristol-Myers Squibb (New York, NY, USA) and bred at the Institut für Medizinische Mikrobiologie, Immunologie und Hygiene, Technische Universität München (Germany). In all experiments reported here, 6- to 10-week-old female mice were used. Age-matched, heterozygous or WT littermates from the same animal room served as controls.

Multiplication and purification of *C. pneumoniae*

C. pneumoniae CM-1 (VR-1360; American Type Culture Collection, Manassas, VA, USA) was multiplied according to Maass and Harig [21]. Chlamydial elementary bodies were centrifuged on confluent monolayers of HEp-2 cells (2000 *g*, 35 min, 35°C) in the presence of cycloheximide (1 μg ml^{-1}) and 0% FBS. After 72 h of culture, the harvested cells were disrupted with glass beads, and chlamydial elementary bodies were purified with a sucrose urografin gradient (bottom layer, 50% w/v sucrose solution; top layer, 30% v/v urografin in 30 mM Tris-HCl buffer, pH 7.4) at 9000 *g* and 4°C for 60 min. After one wash step with 0.2 μm-filtered PBS (pH 7.4), purified elementary bodies were stored in sucrose–phosphate–glutamate buffer (0.22 M sucrose, 8.6 mM Na_2HPO_4, 3.8 mM KH_2PO_4, 5 mM glutamic acid, 0.2 μm-filtered, pH 7.4) at −80°C until use. To quantify the number of elementary bodies, HEp-2 cells were infected and stained with a chlamydia-specific antibody (ACI-FITC, Progen Biotechnik GmbH, Heidelberg, Germany). The number of IFU was counted, as determined by fluorescence microscopy (Carl Zeiss Jena GmbH, Göttingen, Germany), 48 h after infection. Contamination with mycoplasma was excluded regularly by *Mycoplasma*-PCR using specific primers (MWG Biotech, Martinsried, Germany).

Infection of mice with *C. pneumoniae* and treatment with recombinant sgp130-Fc fusion protein

Mice were anesthetized with an i.p. injection of ketamin (2 mg/mouse). Thereafter, mice were infected intranasally with 1.75×10^6 IFU *C. pneumoniae* (CM-1, ATCC VR-1360) in 30 μl PBS. The experiments were performed with the permission of local authorities (Regierung von Oberbayern, Germany; file number 55.2-1-54-2531-59-06). In some experiments, mice were treated 1 day after infection by i.p. injection of 150 μg recombinant sgp130-Fc protein in 200 μl PBS. sgp130-Fc has been described before [22].

Infection of MEFs with *C. pneumoniae* in vitro

To prepare MEFs for infection, the cells were cultured in a 12-well plate at a density of $1.5–2.5 \times 10^5$ cells/well in DMEM (10% FCS). After 6 h, the medium was replaced with DMEM (0% FCS), and MEFs were infected 20 h later with *C. pneumoniae* by centrifugation (3000 rpm, 35 min, 35°C).

Determination of chlamydial load in the lungs

To quantify the pulmonary burden of *C. pneumoniae*, lungs were removed after 3, 6, or 9 days postinfection and were minced to homogeneity in 500 μl PBS as described before [18]. Briefly, HEp2 cells were infected with the prepared lung homogenate, diluted 1:100 in MEMα without FCS containing cycloheximide (1 ng/ml). After 48 h, HEp2 cells were fixed and permeabilized with methanol/acetone (1:1, 5 min) and stained with FITC-labeled mAb specific for chlamydial endotoxin (ACI-FITC, Progen Biotechnik GmbH) and counterstained with Evans blue. Cells were washed and embedded in glycerin/PBS (50%) and analyzed by confocal microscopy (LSM510, Carl Zeiss Jena GmbH).

Lung histology and immunohistochemistry

The formalin-fixed lungs of all *Dusp1*$^{-/-}$ mice and their WT littermates, infected with *C. pneumoniae* and controls, were embedded in paraffin. Sections (3–5 μm-thick) were cut and stained with H&E. Immunohistochemistry was performed on an automated immunostainer (Ventana Medical Systems, Oro Valley, AZ, USA), according to the protocol provided by the company, with minor modifications. The antibody panel used included CD3, MPO (Dako Cytomation, Hamburg, Germany), B220/CD45R, and Mac3 (BD PharMingen, Franklin Lakes, NJ, USA).

Isolation of pulmonary cells, flow cytometry, and FACS sorting

To isolate pulmonary cells, mice were killed by CO_2 inhalation. The lungs were flushed with 10 ml PBS, applied through the right atrium of the heart to remove blood. Thereafter, the organ was cut into small pieces in a 60-mm plate and digested with collagenase VIII (400 U/100 μl, room temperature, C-2139, Sigma, Germany), as described before [17]. The remain-

ing cells were washed in PBS containing 3% FCS and analyzed by FACS. The isolated cells were preincubated with ethidium mono-azide (2 μg/ml, 20 min, 4°C, Molecular Probes, Eugene, OR, USA) to exclude dead cells and anti-CD16/32 (100 μg/ml, 10 min, 4°C) to block FcRs. Subsequently, cells were double-stained (30 min, 4°C) with CD45-PE (2 μg/ml) and Gr1-FITC (5 μg/ml) or triple-stained with CD45-PE (2 μg/ml), F4/80-FITC (2 μg/ml), and CD11b-APC (2 μg/ml). After three wash steps, the cells were fixed with 2% paraformaldehyde, and flow cytometric analysis was performed (BD Biosciences, San Jose, CA, USA). All FACS data were analyzed using Flow Jo (Tree Star Inc., Ashland, OR, USA).

To purify GR1$^+$ and GR1$^-$ cells, we first stained the isolated pulmonary cells with anti-CD16/32 to block FcRs, followed by APC-labeled anti-CD11b mAb, PE-labeled anti-CD45 mAb, and FITC-labeled anti-GR1 mAb. Cells were gated on CD45 expression, and CD11b$^+$/GR1$^+$ and CD11b$^+$/GR1$^-$ subsets were sorted with a MoFlow instrument (Dako Cytomation).

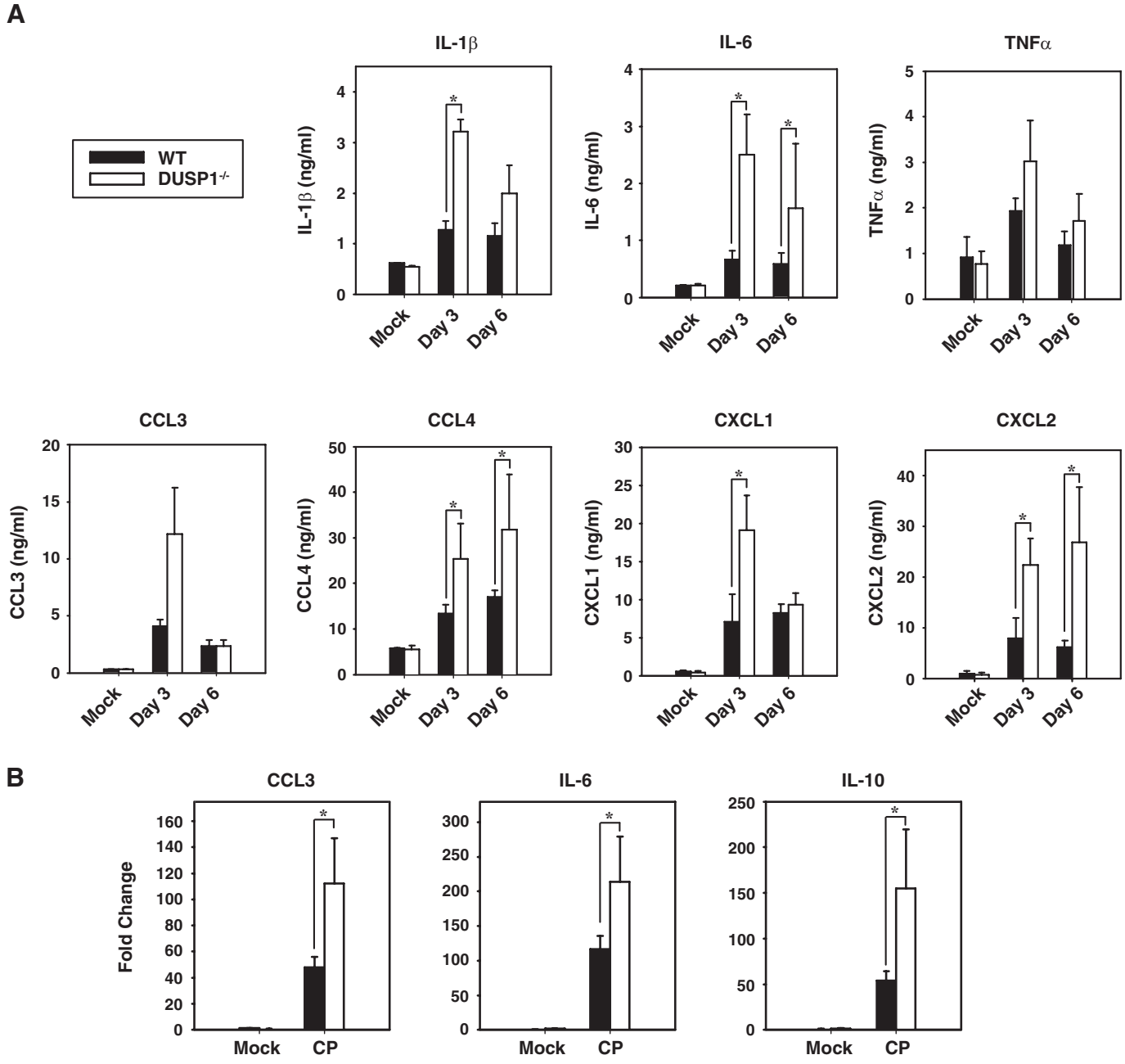

Figure 1. Increased inflammatory response to *C. pneumoniae* in the lungs of *Dusp1*$^{-/-}$ mice. (A) Cytokine and chemokines at the protein level in supernatants of minced lungs are increased. WT and *Dusp1*$^{-/-}$ mice (solid and open bars, respectively) were inoculated intranasally with PBS as control (Mock) or infected with *C. pneumoniae* for 3 or 6 days. The levels of CXCL1, CXCL2, CCL3, CCL4, TNF-α, IL-1β, and IL-6 were determined by ELISA [Mock, n=2–4; *C. pneumoniae* (CP), n=4–7]. Error bars represent the SD of equally treated mice. *, $P \leq 0.05$, Student's t-test. (B) qRT-PCR analysis of inflammatory gene expression in the lungs of infected (CP) or mock-infected mice (Mock) at Day 3 after infection. Mean and SD of pooled data from two experiments (Mock, n=4; CP, n=6/genotype). *, $P \leq 0.05$, Student's t-test.

ELISA for chemokine and cytokine production

Levels of the chemokines CXCL1, CXCL2, CCL3, and CCL4, as well as the cytokines TNF-α, IL-1β, IL-6, IFN-γ, and IL-12p40, and the sIL-6Ra were determined in supernatants of homogenized lungs of mice by commercially available ELISAs (Duo Set, R&D Systems, Wiesbaden-Nordenstadt, Germany). The assays were performed as recommended by the manufacturer.

RNA isolation and qRT-PCR

Mice were killed by CO_2 inhalation 3 days postinfection with *C. pneumoniae*. The lungs were perfused to remove blood cells as described above. Immediately thereafter, lungs were transferred to precooled tubes placed in an ethanol/dry ice bath, and after homogenization, RNA was isolated using TriFAST (Peqlab, Germany) as described by the manufacturer. qRT-PCR was performed in duplicates using cDNA reverse-transcribed from 1 μg lung RNA with a combined oligo-$dT_{(12–18)}$/random hexamer-primed first-strand synthesis. The Roche Universal Probe Library system was used to select specific primers and probes. Sequences are available upon request from the authors. PCR was carried out for 45 cycles. Data were processed according to the DDCT method, using hypoxanthine-guanine phosphoribosyltransferase as a housekeeping gene for normalization and the mock-infected WT lung RNA as a calibrator for calculation of fold changes.

RESULTS

Increased pulmonary inflammation in *Dusp1*$^{-/-}$ mice after infection with *C. pneumoniae*

During *C. pneumoniae* infection, TLR2 and TLR4 play an important role in triggering cytokine and chemokine production in the lung [17]. Mice deficient in the MAPK phosphatase DUSP1 respond to TLR ligation in vivo with overshooting release of a subset of cytokines. We investigated the role of DUSP1 in controlling the inflammatory response at different time-points after intranasal infection with *C. pneumoniae*. At Day 3 postinfection, *Dusp1*$^{-/-}$ mice had two- to threefold higher levels of the chemokines CCL3, CCL4, CXCL1, and CXCL2 in lung homogenates compared with the WT mice (**Fig. 1A**). The secretion of IL-1β and IL-6 was also elevated signifi-

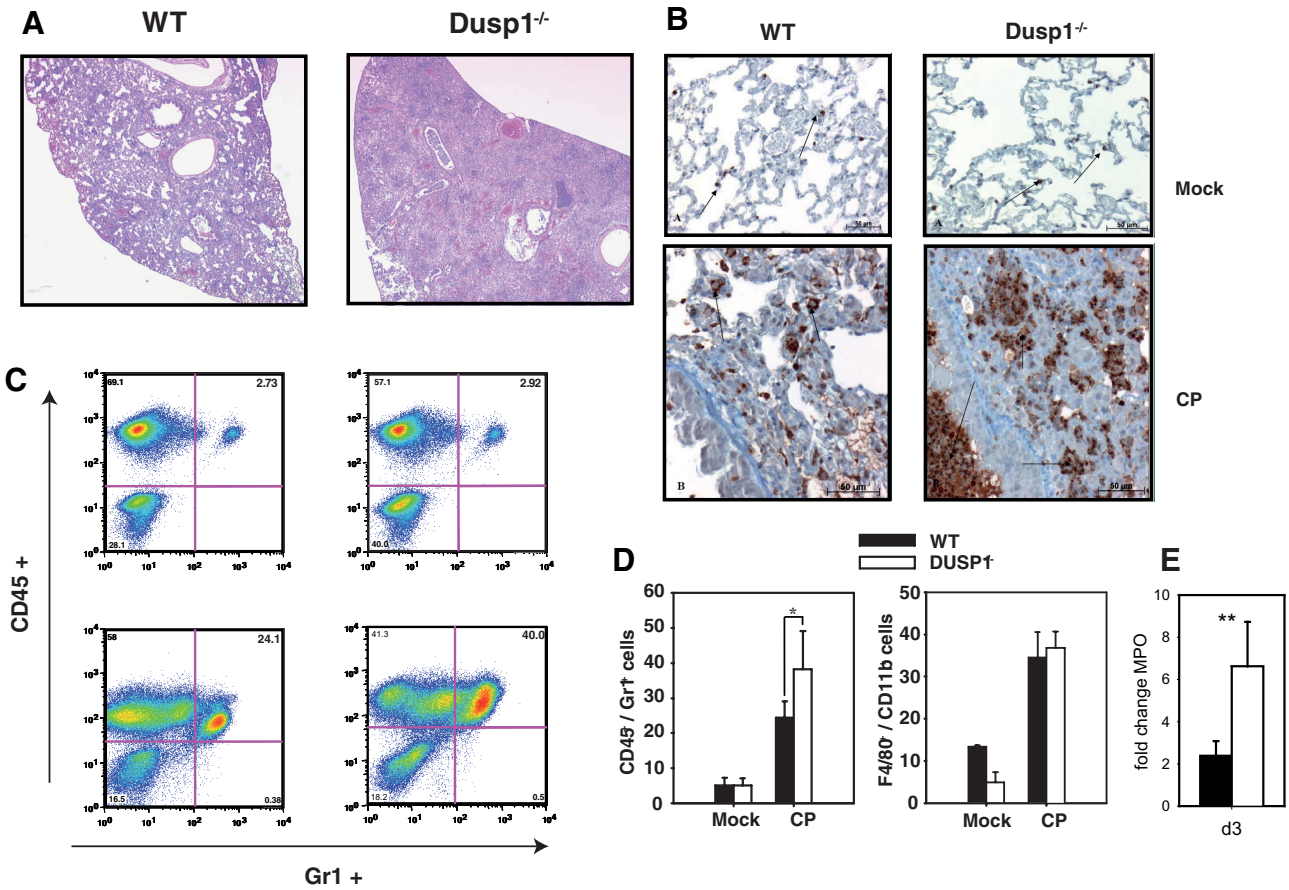

Figure 2. Infiltration of leukocytes in *C. pneumoniae*-infected *Dusp1*$^{-/-}$ mice. (A) H&E staining of lung sections from WT (left) and Dusp1$^{-/-}$ (right) mice 3 days after infection. Overview of lung tissue from representative mice. (B) MPO staining of lung sections from control (Mock) or infected mice (CP). Positive cells are indicated (arrows). Original size bars indicate magnification. (C) Higher recruitment of Gr1$^+$ cells into infected lungs of *Dusp1*$^{-/-}$ mice. Lungs of *C. pneumoniae*-infected mice (CP, lower graphs) as well as controls (Mock, upper graphs) were digested with collagenase as described above. Single-cell suspensions were stained for Gr1 and CD45. (D) Percentage of granulocytes and macrophages in lungs of WT and *Dusp1*$^{-/-}$ mice after infection. The graphics represent the average from different experiments for the populations of cells expressing CD45$^+$/Gr1$^+$ or F4/80$^+$/CD11b$^+$ (gated on CD45$^+$) in lungs of mock (n=4 WT and *Dusp1*$^{-/-}$) or infected (CP, n=5–6, both genotypes) mice. *, $P < 0.05$. (E) qRT-PCR analysis of MPO expression in the lungs 3 days (d3) after infection. Fold changes relative to uninfected controls were calculated by the DDCT method. Mean and SD (n=4); **, $P < 0.01$, t-test.

cantly in the absence of DUSP1, and TNF-α levels were not increased significantly (Fig. 1A). Similar observations were made when lung mRNA was analyzed for expression of *Ccl3* and *Il6* by qRT-PCR at Day 3 after infection (Fig. 1B). IL-10 protein could not be detected by ELISA in the lung after infection but was clearly induced more strongly by *C. pneumoniae* infection in *Dusp1*$^{-/-}$ lungs (Fig. 1B). At Day 6 postinfection, *Dusp1*$^{-/-}$ mice still showed increased production of IL-6, CCL4, and CXCL2. However, the increased secretion of IL-1β, CCL3, and CXCL1 was transient, observing comparable levels in both genotypes at Day 6 (Fig. 1A).

We next performed histological analysis of the lungs. Under basal conditions, *Dusp1*$^{-/-}$ mice showed no pulmonary pathology (not shown). In both genotypes, analysis of mice 3 days after chlamydial infection showed infiltration by polymorphonuclear cells. Consistent with the elevated chemokine production, the degree of leukocyte infiltration was histologically more severe in *Dusp1*$^{-/-}$ mice (**Fig. 2A**). In contrast to the interstitial pneumonia observed in infected WT mice, in the absence of DUSP1, acute bronchopneumia, with more pronounced destruction of the lung parenchyma, was evident. A MPO staining of the sections confirmed that the majority of infiltrating cells was granulocytes (Fig. 2B). In addition, expression of the *Mpo* gene encoding MPO was increased more strongly in *Dusp1*$^{-/-}$ lung tissue mRNA (Fig. 2E). Immunohistochemistry showed comparable infiltration with Mac3^{+} macrophages in the infected mice (not shown). Independent analysis of collagenase-digested lungs by flow cytometry confirmed that the percentage of Gr1^{+} leukocytes was higher in *Dusp1*$^{-/-}$ mice after infection, whereas the percentage of CD11b^{+}F4/80^{+} macrophages was comparable between infected WT and *Dusp1*$^{-/-}$ mice (Fig. 2, C and D).

Increased pulmonary chlamydial load and weight loss in the absence of DUSP1

To test the assumption that the more robust inflammatory response in *Dusp1*$^{-/-}$ mice may facilitate the rapid control of the invading microorganism, the impact of *Dusp1* deficiency on chlamydial replication was determined in parallel to the chemokine/cytokine measurements from lung homogenates of infected mice. Contrary to our expectation, we found that *Dusp1*$^{-/-}$ mice had a significant two- to threefold increase in pulmonary chlamydial burden at Days 3 and 6 after infection (**Fig. 3A**). Similar to the dysregulated response of some chemokines and cytokines, this difference was transient, as *Dusp1*$^{-/-}$ mice, at Day 9, controlled the infection comparable with WT mice (Fig. 3A). As an additional measure of clinical disease, we determined the body weight of infected mice over time and found a significant, but again, transient aggravation of weight loss in *Dusp1*$^{-/-}$ compared with WT mice (Fig. 3B). Consistent with the regaining of weight in *Dusp1*$^{-/-}$ mice, all mice of both genotypes survived the infection (data not shown). Together, these data showed an impaired, early resistance of *Dusp1*$^{-/-}$ mice to *C. pneumoniae* infection despite an increased innate inflammatory response.

Expression of IFN-γ-induced, antichlamydial effector molecules

The dissociation of inflammatory cytokine production from antichlamydial resistance in *Dusp1*$^{-/-}$ mice suggested that the production or effects of IFN-γ [12] might be compromised in the absence of this MAPK phosphatase. To address this possibility, we analyzed

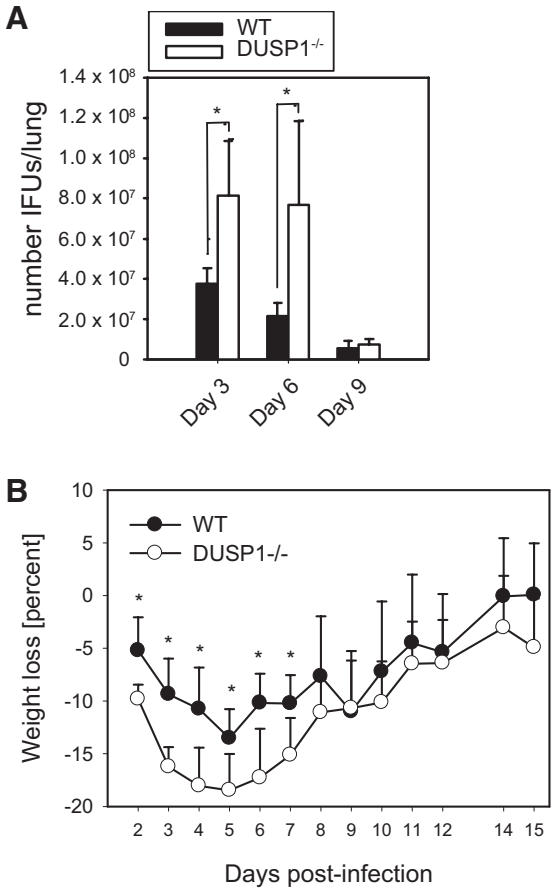

Figure 3. Higher chlamydial load and weight loss in *Dusp1*$^{-/-}$ mice. (A) Chlamydial burden in lungs 3, 6, and 9 days postinfection from WT (Day 3, n=6; Day 6, n=9; Day 9, n=8) and *Dusp1*$^{-/-}$ mice (Day 3, n=8; Day 6, n=9; Day 9, n=8). Chlamydial burden was quantified by transfer of lung supernatants to HEp2 cells. Forty-eight hours later, IFU in HEp2 cells were counted. *, $P \leq 0.05$, t-test . (B) *Dusp1*$^{-/-}$ mice lose more body weight during infection with *C. pneumoniae*. Mice were infected with *C. pneumoniae* and were measured every day. The graphic represents the percent loss of body weight relative to Day 0. WT and *Dusp1*$^{-/-}$ mice (n=5–7). Error bars represent the SD of equally treated mice. *, $P \leq 0.05$, Student's t-test. The weight of uninfected mice at Day 0 did not differ significantly (WT, 18.1±3.4 g; *Dusp1*$^{-/-}$, 19.8±1.8 g).

the expression of IFN-γ, its inducers, and downstream mediators, which are essential for control of chlamydial growth in vivo [23]. Although the average levels of IL-12 were diminished in infected *Dusp1*$^{-/-}$ lungs, this difference was not statistically significant, and production of IFN-γ was not altered significantly (**Fig. 4A**). Among the multitude of IFN-γ-induced genes, the NO-generating enzyme iNOS and the 47-kDa GTPases *Irgm*, *Igtp*, *Iigp2*, and *Irgb10* have been demonstrated to play an important role in restricting chlamydial growth [13, 14, 24]. Of these antichlamydial effector molecules, only the induction of *Igtp* and *Iigp2* expression was diminished significantly but modestly in *Dusp1*$^{-/-}$ lungs; in contrast, iNOS mRNA expression was induced to higher levels than in WT mice (Fig. 4B). Collectively, these gene expression data suggest that the induction of antichlamydial effector programs downstream of IFN-γ is largely intact in *Dusp1*$^{-/-}$ mice.

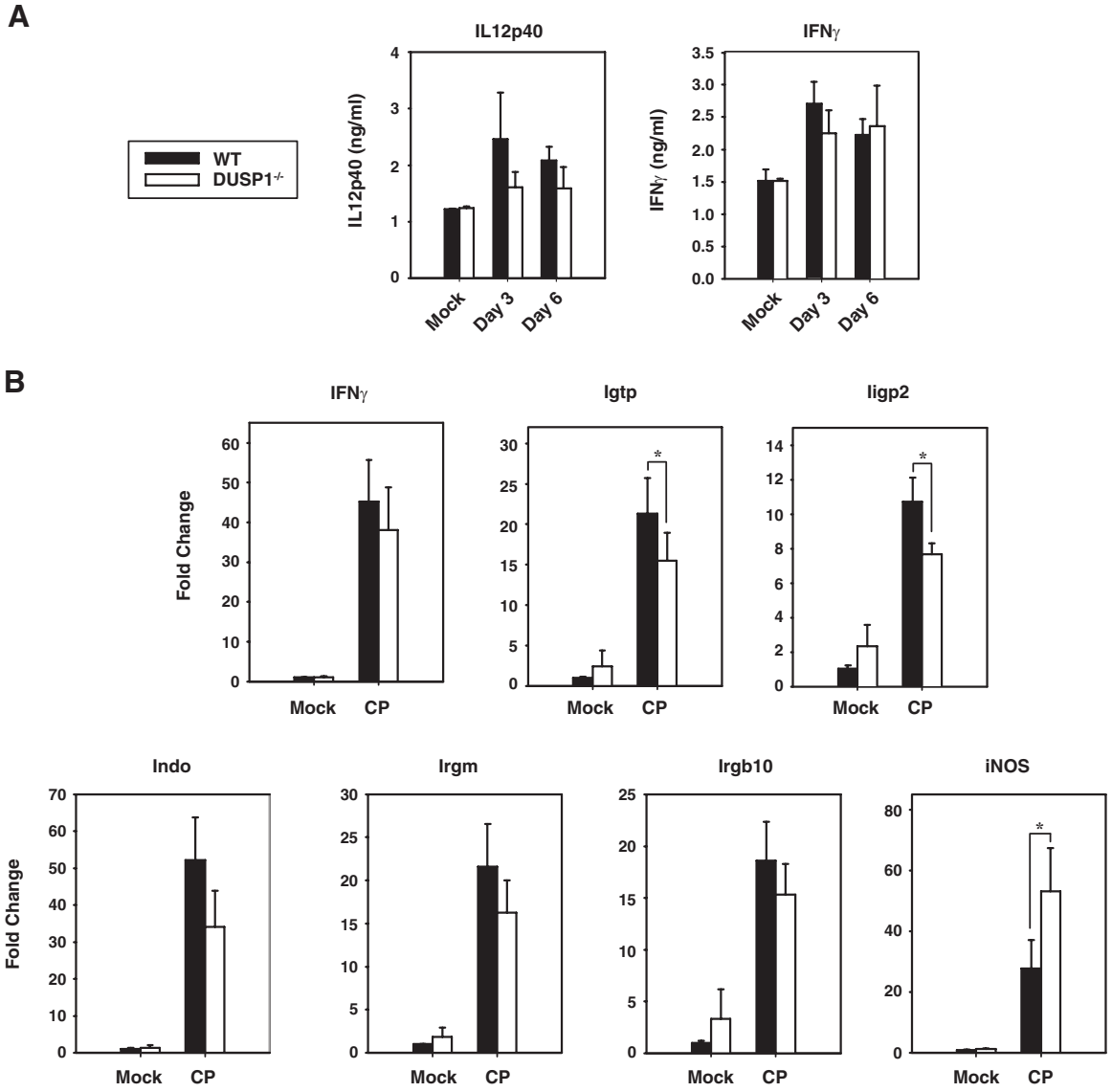

Figure 4. Expression of IL-12, IFN-γ, and antichlamydial effector molecules. (A) WT and *Dusp1*$^{-/-}$ mice were infected with *C. pneumoniae* or not infected as control (Mock), and the levels of IL-12p40 and IFN-γ were quantified by ELISA after 3 and 6 days of infection (Mock, n=2; CP, n=4–9, both genotypes) Error bars represent the SD of equally treated mice. (B) qRT-PCR analysis of IFN-γ target gene expression in the lungs of infected (CP) or mock-infected mice (Mock) at Day 3 after infection. Mean and SD of pooled data from two experiments (Mock, n=4; CP, n=6/genotype). *, $P \leq 0.05$, Student's t-test.

Blockade of IL-6 trans-signaling corrects hyperinflammation and increased chlamydial load in Dusp1$^{-/-}$ mice

An alternative mechanism behind the increased chamydial burden in *Dusp1*$^{-/-}$ lungs could be the enhancement of bacterial replication by inflammatory factors. Of interest, in earlier work addressing the role of the TLR adaptor protein MyD88 in *C. pneumoniae* infection, a correlation of reduced chlamydial replication and dampened inflammation in MyD88$^{-/-}$ mice was observed. This link could be explained by a replication-enhancing effect of neutrophils on the bacterium [18], whose molecular basis is currently unknown. Among the cytokines controlled by DUSP1 in chlamydial infections, but also in gram-positive infection and during LPS shock, is IL-6, whose pleiotropic effects include the recruitment and activation of leukocytes. A complex of IL-6 with the soluble form of the IL-6Ra can bind to the signaling receptor chain gp130, which is expressed on many cell types. This process is termed IL-6 trans-signaling and renders IL-6Ra-negative cells, such as endothelial cells, IL-6-responsive [25]. Of interest, we found that the sIL-6R was up-regulated in the lung following *C. pneumoniae* infection, and *Dusp1*$^{-/-}$ mice had an exaggerated response at Day 3 (**Fig. 5A**). As the components of IL-6 trans-signaling were therefore overabundant in *Dusp1*$^{-/-}$ mice after infection, we used a fusion protein of soluble gp130 with a Fc fragment (sgp130-Fc) to block IL-6 trans-signaling [22] in vivo and to test its contribution to inflammation and impaired resistance in *C. pneumoniae* infection (Fig. 5, B and C). A single injection of

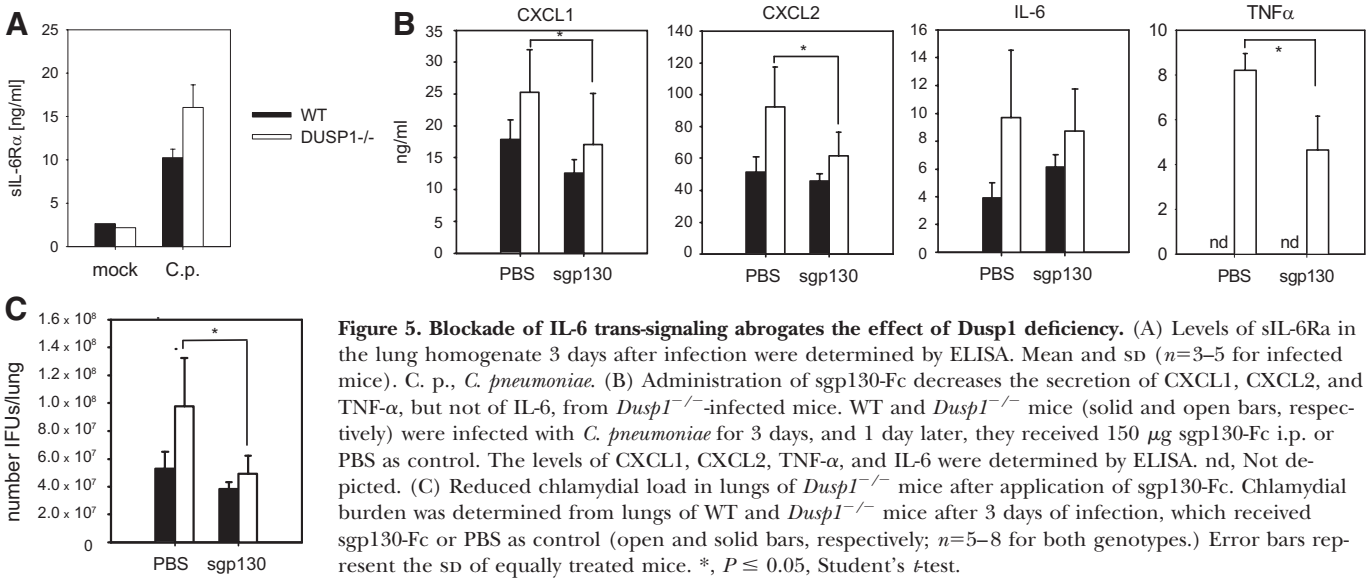

Figure 5. Blockade of IL-6 trans-signaling abrogates the effect of Dusp1 deficiency. (A) Levels of sIL-6Ra in the lung homogenate 3 days after infection were determined by ELISA. Mean and SD (n=3–5 for infected mice). C. p., *C. pneumoniae*. (B) Administration of sgp130-Fc decreases the secretion of CXCL1, CXCL2, and TNF-α, but not of IL-6, from *Dusp1*$^{-/-}$-infected mice. WT and *Dusp1*$^{-/-}$ mice (solid and open bars, respectively) were infected with *C. pneumoniae* for 3 days, and 1 day later, they received 150 μg sgp130-Fc i.p. or PBS as control. The levels of CXCL1, CXCL2, TNF-α, and IL-6 were determined by ELISA. nd, Not depicted. (C) Reduced chlamydial load in lungs of *Dusp1*$^{-/-}$ mice after application of sgp130-Fc. Chlamydial burden was determined from lungs of WT and *Dusp1*$^{-/-}$ mice after 3 days of infection, which received sgp130-Fc or PBS as control (open and solid bars, respectively; n=5–8 for both genotypes.) Error bars represent the SD of equally treated mice. *, $P \leq 0.05$, Student's t-test.

sgp130-Fc 1 day postinfection reduced the levels of CXCL1, CXCL2, and TNF-α in the lungs of *Dusp1*$^{-/-}$ mice, whereas IL-6 levels were not affected. Of note, sgp130-Fc injection increased IL-6 levels in the serum of WT mice, probably as a result of an increased half-life of the complex, as reported during therapy with anti-IL-6 antibodies [26]. Thus, similar to models of sterile inflammation [27, 28], increasing the levels of sgp130 caused a reduction of chemokine and cytokine production in the setting of *C. pneumoniae* infection in *Dusp1*$^{-/-}$ mice (Fig. 5B). Blockade of IL-6 trans-signaling also had a clear effect on chlamydial replication, reducing the bacterial load in the lungs of *Dusp1*$^{-/-}$ mice to the level observed in WT mice (Fig. 5C). Thus, dysregulated IL-6 production in the absence of DUSP1 plays an important role in propagating pulmonary inflammation and enhancing chlamydial proliferation.

IL-6 is produced mainly by granulocytes and enhances chlamydial replication directly

Given our earlier observation that granulocytes enhance replication of *C. pneumoniae in vitro* [18] and the increased chlamydial numbers as a result of IL-6 trans-signaling in the lung, we asked whether granulocytes are the source of this cytokine in *C. pneumoniae* infection. Indeed, when FACS-sorted cells from the lungs of infected mice were investigated for spontaneous production of IL-6, we found that neutrophils produce much higher levels of IL-6 compared with CD11b^{+}Gr1^{-} macrophages (**Fig. 6A**). On a per-cell basis, the release of IL-6 was comparable between WT and *Dusp1*$^{-/-}$ cells; however, the higher number of granulocytes in the lungs of infected *Dusp1*$^{-/-}$ mice (Fig. 2) explains the higher levels of IL-6 measured in the lung homogenates (Fig. 1). We next performed a direct test of the effects of IL-6 on chlamydial replication using MEFs in vitro (Fig. 6B). IL-6 dose-dependently increased the number of chlamydial IFU with a peak at a concentration of 10 ng/ml. Of interest, the timing of IL-6 addition to the cells had a major influence on the magnitude of the effect, with a maximal increase in IFU numbers when IL-6 was added directly after bringing chlamydia and cells together by centrifugation (Fig. 6C).

DISCUSSION

Our analysis of *C. pneumoniae* infection in *Dusp1*$^{-/-}$ mice revealed a differential regulation of inflammation and control of chlamydial growth by this MAPK phosphatase. As *C. pneumoniae* activates TLR2 and TLR4 [29], the increased inflammatory response in the absence of DUSP1 was not unexpected, given previous results in LPS challenge models [5, 6, 8] and infection with *S. aureus* or *Escherichia coli* [9, 30]. In addition, a similar subset of target genes was controlled by DUSP1 in these models, with overshooting expression of many chemokines and inflammatory cytokines in the knockout mice. The higher bacterial load in *Dusp1*$^{-/-}$ mice shows that in infection with *C. pneumoniae*, an increased innate inflammatory response per se does not confer better resistance. This separation may be explained by the lack of enhancement of IFN-γ production and target gene expression in *Dusp1*$^{-/-}$ mice. On the other hand, the normal expression of IFN-γ-induced antimicrobial effector molecules also indicates that the increased chlamydial replication is not caused by a suppression of this pathway in the absence of DUSP1. Indeed, we demonstrate that overactive production of IL-6 is likely responsible for the increased bacterial load via trans-signaling and enhancement of chlamydial replication. This higher bacterial load in *Dusp1*$^{-/-}$ mice may then stimulate the inflammatory response further.

During the preparation of this manuscript, two studies were published that examined the role of other endogenous regulators of innate immunity in *C. pneumoniae* infection. In the absence of the immunosuppressive cytokine IL-10, chlamydial infection triggered more severe inflammation, which was associated with accelerated clearance of the bacterium [31]. *Dusp1* expression is induced by IL-10 [32] and probably mediates part of its effects. From the data reported here, it appears that DUSP1 contributes to the down-regulation of inflammatory gene expression but is not involved in the impairment of antichlamydial effector function by IL-10. Consistent with this interpretation is the strong increase in pulmonary IFN-γ levels in the *Il10*$^{-/-}$ mice [31], which is not seen in the absence of

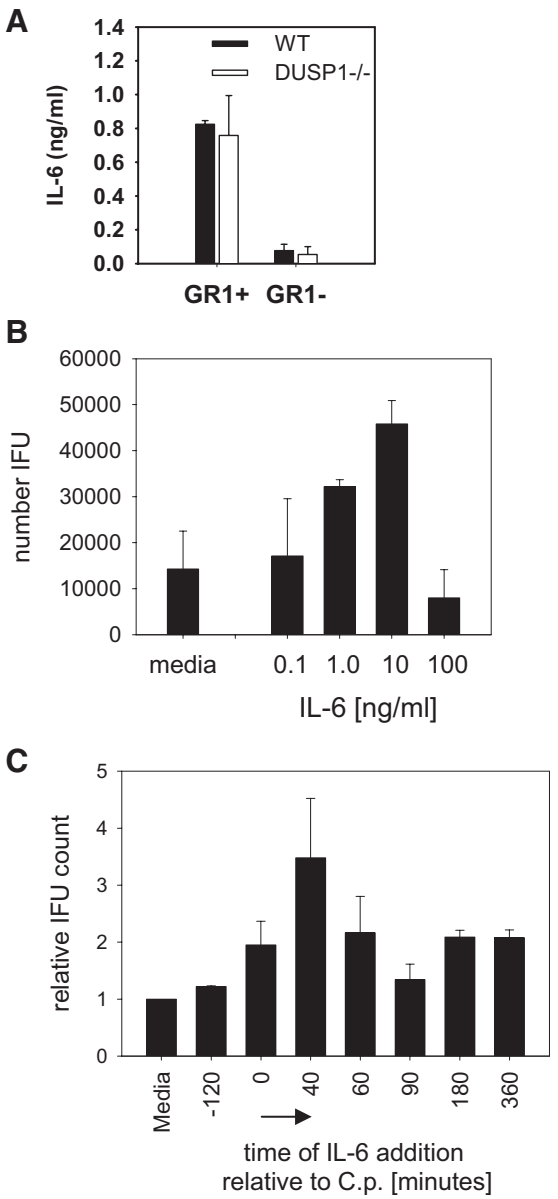

Figure 6. IL-6 enhances chlamydial replication directly. (A) $CD45^+Gr1^+$ granulocytes or $CD45^+Gr1^-$ cells were FACS-sorted from collagenase-digested lung cells and plated at a density of 1.0×10^5 cells/well on a monolayer of MEF in 12-well plates. Supernatants were collected after 48 h and analyzed by ELISA. Mean and SD from two independent experiments. (B) MEFs (2×10^5 cells/well) were infected in 12-well plates with *C. pneumoniae* at a MOI of 3. IL-6 was added at the indicated concentrations after the centrifugation step to infect the cells. After 48 h, cells were lysed and the number of IFU determined as described [18]. (C) IL-6 was added to MEFs at a concentration of 20 ng/ml at the indicated time-points relative to infection with *C. pneumoniae* (MOI=2). The arrow indicates centrifugation for 35 min to synchronize infection. Chlamydial IFU were normalized to infection in the absence of IL-6. Mean and SD of three experiments (medium, 40, 90, and 360 min) or two experiments (–120, 0, 60, and 180 min).

DUSP1. It should be noted that *Dusp1*$^{-/-}$ not only have increased levels of inflammatory cytokines but also express significantly more IL-10 upon infection with *C. pneumoniae* (this study), *S. aureus* [9], and *E. coli* [30], as well as after LPS injection [5, 6, 8]. In the case of *E. coli* infection, the impaired control of bacterial growth in *Dusp1*$^{-/-}$ mice was restored when the mice were back-crossed onto an *Il10*$^{-/-}$ background [30]. It is therefore possible that increased IL-10 contributes to the imparied control of chlamydial replication in the lungs of *Dusp1*$^{-/-}$ mice. This possibility was not explored here, as we focused on the role of IL-6 (see below), which was readily detected in the lungs at the protein level. The effect of a deficiency in SOCS-1, a regulator of IFN-γ signaling, was investigated by Yang et al. [33], who found rapid clearance of *Chlamydia* coupled to overshooting IFN-γ target gene expression; as IL-6 expression was normal in *Socs1*$^{-/-}$ mice, it shows a phenotype that is reciprocal to what we observed in *Dusp1*$^{-/-}$ mice. Together, the studies demonstrate nicely the complexity and specificity of negative regulators targeting different pathways of macrophage activation and deactivation in balancing the host response to infection with *C. pneumoniae*.

That enhanced neutrophil infiltration in *Dusp1*$^{-/-}$ mice correlates with increased chlamydial load is consistent with our earlier data showing direct enhancement of *C. pneumoniae* replication by granulocytes [18] and recent data from *Chlamydia psittaci* infection demonstrating that neutrophil recruitment is required for susceptibility [14]. Importantly, we have identified DUSP1-regulated IL-6 production and IL-6 trans-signaling as causal for amplifying the chemokine response that is responsible for leukocyte recruitment to the lungs. The importance of IL-6 and IL-6 trans-signaling for the recruitment of neutrophils has been demonstrated before in models of arthritis and kidney injury [27, 34]. Shedding of the IL-6R from neutrophilic granulocytes has been described [35] and may contribute further to inflammatory trans-signaling in *C. pneumoniae* infection in *Dusp1*$^{-/-}$ mice. Our data also show that granulocytes are the major source of IL-6 in *C. pneumoniae*-infected lungs. In fact, as IL-6 alone had an enhancing effect on chlamydial replication in vitro, this cytokine is likely the active principle behind the prochlamydial effect of granulocytes in vitro and in vivo [18]. How exactly IL-6 promotes the growth of chlamydia remains to be determined. Possible scenarios to be tested in future experiments include increased availability of nutrients (e.g., amino acids, iron, ATP) in IL-6-treated cells [36, 37] or inhibitory effects of IL-6 on the cell-autonomous, antichlamydial defense mechanisms, such as indoleamine-2,3-deoxygenase [37].To the best of our knowledge, effects of inhibition of IL-6 trans-signaling in an infectious disease model have not been reported before. As antibody therapies targeting IL-6R signaling are entering the clinic for long-term treatment of chronic inflammatory disorders, their effects on the host response to intermittent bacterial infections will be important to monitor.

AUTHORSHIP

N.R. performed most experiments, analyzed data, and contributed to study design and writing of the manuscript. H.D.,

G.W., and M.H. performed experiments. I.M. and L.Q-M. provided histological analyses. J.S. and S.R-J. provided critical reagents. T.M. contributed to study design and writing of the manuscript and analyzed data. R.L. designed the study, analyzed data, and wrote the manuscript.

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