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Lung-Specific Overexpression of CC Chemokine Ligand (CCL) 2 Enhances the Host Defense to *Streptococcus pneumoniae* Infection in Mice: Role of the CCL2-CCR2 Axis

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Mononuclear phagocytes are critical components of the innate host defense of the lung to inhaled bacterial pathogens. The monocyte chemotactic protein CCL2 plays a pivotal role in inflammatory mononuclear phagocyte recruitment. In this study, we tested the hypothesis that increased CCL2-dependent mononuclear phagocyte recruitment would improve lung innate host defense to infection with *Streptococcus pneumoniae*. CCL2 transgenic mice that overexpress human CCL2 protein in type II alveolar epithelial cells and secrete it into the alveolar air space showed a similar proinflammatory mediator response and neutrophilic alveolitis to challenge with *S. pneumoniae* as wild-type mice. However, CCL2 overexpressing mice showed an improved pneumococcal clearance and survival compared with wild-type mice that was associated with substantially increased lung mononuclear phagocyte subset accumulations upon pneumococcal challenge. Surprisingly, CCL2 overexpressing mice developed bronchiolitis obliterans upon pneumococcal challenge. Application of anti-CCR2 Ab MC21 to block the CCL2-CCR2 axis in CCL2 overexpressing mice, though completely abrogating bronchiolitis obliterans, led to progressive pneumococcal pneumonia. Collectively, these findings demonstrate the importance of the CCL2-CCR2 axis in the regulation of both the resolution/repair and remodelling processes after bacterial challenge and suggest that overwhelming innate immune responses may trigger bronchiolitis obliterans formation in bacterial lung infections. *The Journal of Immunology*, 2007, 178: 5828–5838.

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Materials and Methods

Animals

CCL2 overexpressing mice harboring an endogenous human ccl2 transgene under the control of the surfactant protein C promoter selectively expressed by type II alveolar epithelial cells were generated as previously described (4) and backcrossed eight generations onto the BALB/c background. Wild-type BALB/c mice (Charles River Breeding Laboratories) were used as controls. Mice were used in all the experiments at 8–12 wk of age in accordance with the guidelines of the Institutional Animal Care and Use Committee for the Hannover School of Medicine (Hannover, Germany). Animal experiments were approved by our local government authorities.

Reagents

Abs used in the current study for mononuclear phagocyte subset-specific analysis were purchased from BD Biosciences or Serotec as indicated. CCR2 inhibition studies were performed using the function-blocking rat anti-mouse anti-CCR2 mAb MC21, which was recently shown to be highly effective in blocking inflammatory mononuclear phagocyte immigration into the lungs of mice (3, 5).

Culture and quantification of S. pneumoniae and infection of CCL2 overexpressing mice and wild-type mice

Pneumolysin-producing clinical isolate of the S. pneumoniae capsular group 19 strain EF3030 was grown in Todd-Hewitt broth (Difco) supplemented with 0.1% yeast extract to mid-log phase. Prepared aliquots were snap-frozen in liquid nitrogen and stored at −80°C until use, as outlined in detail recently (6). Pneumococci were quantified by plating serial dilutions of the bacteria on sheep blood agar plates (BD Biosciences) followed by incubation of the plates at 37°C/5% CO2 for 18 h and subsequent determination of CFU by eye.

Infection of CCL2 overexpressing and wild-type mice with S. pneumoniae was done using freshly prepared dilutions of thawed aliquots adjusted to −3 × 10^7 CFU/mouse, as recently described (3). Briefly, tracheas were exposed by surgical resection, and intratracheal instillation of the pneumococci was performed under stereomicroscopic control (MS 5; Leica) using a 26-gauge catheter (Abbocath) inserted into the trachea. After instillation, the neck wound was closed with sterile sutures.

For CCR2 inhibition studies, mice received sterile i.p. injections of the function-blocking anti-CCR2 mAb MC21 (100 μg/mouse) 3 h before and every 24 h subsequent to intratracheal application of S. pneumoniae for the 7-day observation period. MC21-treated wild-type mice were infected with −2 × 10^7 CFU/mouse to allow the mice to survive the observation period of 7 days. In all experiments, mice were kept under specific pathogen-free conditions with free access to autoclaved food and water and were monitored twice daily for disease symptoms during the entire observation period.

Determination of bacterial loads in the lungs of CCL2 overexpressing and wild-type mice

In line with a recent report (6), our initial experiments showed that >90% of bacterial loads in the lungs of serotype 19 S. pneumoniae-infected mice of either treatment group were accessible by bronchoalveolar lavage (BAL). Therefore, CFU within the lungs of S. pneumoniae-infected CCL2 overexpressing and wild-type mice were determined from whole lung washes. Briefly, mice were euthanized with an overdose of isoflurane. Tracheas of the mice were exposed and cannulated with a shortened 20-gauge needle that was firmly fixed to the trachea. Subsequently, 300-μl aliquots of ice-cold sterile PBS were instilled and subsequently aspirated until a first BAL fluid volume of 1.5 ml was collected. Subsequently, BAL was continued until an additional BAL fluid volume of 4.5 ml was collected. The 1.5 and 4.5 ml BAL fluid samples (whole lung washes) collected from control mice or S. pneumoniae-infected mice of either treatment group were immediately processed for determination of bacterial loads by plating 100 μl of the respective BAL fluid aliquots in 10-fold serial dilutions on sheep blood agar plates followed by incubation of the plates at 37°C/5% CO2 for 18 h. Subsequently, CFU were counted and bacterial loads in whole lung washes were calculated. Whole lung washes were further subjected to centrifugation at 1400 rpm (4°C, 10 min), and cell pellets were pooled to determine the total number of BAL fluid leukocytes. In addition, BAL fluid cytokines were measured in cell-free BAL fluid supernatants of the respective 1.5-ml BAL fluid aliquots.

Mice were euthanized with an overdose of isoflurane (Forene; Abbott). Collection of BAL for the isolation of resident alveolar macrophages and alveolar recruited leukocytes from untreated and S. pneumoniae-infected CCL2 overexpressing and wild-type mice was done as described in detail recently (7, 8). The quantification of BAL fluid neutrophils was done on differential cell counts of Pappenheim-stained cytocentrifuge preparations, using overall morphologic criteria, including cell size and shape of nuclei and subsequent multiplication of those values by the respective absolute BAL cell counts (7, 8). Quantification of resident and recruited mononuclear phagocyte subsets (alveolar macrophages, alveolar dendritic cells (DC), and exudate monocytes/macrophages) recovered by BAL from the lungs of wild-type mice and CCL2 overexpressing mice, respectively, was done using FACS-based differences in immunophenotypic profiles discriminating these mononuclear phagocyte subsets, including differences in their cell surface Ag expression profiles of CD11b, CD11c, MHC class II (MHC-II), CD86, and CD49d, as outlined below in detail and elsewhere (9).

To quantify mononuclear phagocyte subsets in lung parenchymal tissue of uninfected CCL2 overexpressing and wild-type mice, animals were subjected to BAL as described, followed by careful perfusion of the lungs via the right ventricle with HBSS until lung lobes were visually free of blood, as previously described (9). Briefly, lung lobes were carefully removed while avoiding contaminations with lymphatic tissue or conducting airways and then cut into small pieces and incubated in digestion solution consisting of RPMI 1640 supplemented with collagenase A and D (500 kU/ml) for 90 min at 37°C. After incubation, the digested tissue was further disrupted by gently pipetting with a 1-ml syringe and then passed through 100- and 40-μm cell strainers (BD Biosciences) until digestion was finally stopped by adding RPMI 1640/10% FCS. Leukocyte subsets contained in lung homogenates were further purified using a CD45 MACS kit following the instructions of the manufacturer (Miltenyi Biotec). Briefly, the cells were spun at 1200 rpm for 10 min at 4°C and the pellet was resuspended in MACS buffer. After a brief centrifugation step, the cells were incubated with Octagam (10 μl of octagam/10^7 cells; Octapharma) on ice for 10 min to block nonspecific Ab binding, then washed with MACS buffer and incubated with CD45 beads (10 μl of beads/10^7 cells) for 15 min at 4°C. After incubation, cells were washed, centrifuged, and passed over a MACS MS column that was gently flushed with MACS buffer to purify the CD45-positive cells (~90% purity), which were then subjected to FACS analysis of differential cell surface Ag expression profiles, as outlined below.

Immunophenotypic analysis of mononuclear phagocyte subsets in BAL and lung parenchymal tissue

Mononuclear phagocyte subset populations contained in the BAL fluid and lung parenchymal tissue of untreated or S. pneumoniae-challenged CCL2 overexpressing mice and wild-type mice were subjected to flow cytometric immunophenotypic analysis of their cell surface Ag expression profiles. Cells preincubated with octagam were stained for 15 min at 4°C with various combinations of appropriately diluted fluorochrome-conjugated mAbs specific for the following cell surface Ag expression markers (9): Cy5.5-conjugated anti-CD11b, PE Cy5.5- or allophycocyanin-conjugated anti-CD11c, PE-conjugated anti-CD86, PE Cy7- or allophycocyanin-conjugated anti-Gr-1, PE-conjugated anti-MHC-II Ab (all from BD Biosciences), and allophycocyanin-conjugated anti-CD4-F4/80 (Serotec), as most recently described. Subsequently, cells were washed in PBS/0.1% BSA/0.02% sodium azide, and cell acquisition was performed on a FACSCanto flow cytometer (BD Biosciences) equipped with an argon ion laser operating at 488 nm excitation wavelength and a helium neon laser operating at 633 nm wavelength. Gating of the respective mononuclear phagocyte subsets was done according to their forward light scatter (FSC)-A vs side light scatter (SSC)-A characteristics and FSC-A vs F4/80-allophycocyanin fluorescence emission characteristics to exclude contaminating neutrophils from further analysis. Data analysis and careful post-acquisition compensation of spectral overlaps between the various fluorescence channels was performed using FACSDiva software (BD Biosciences).

Lung histopathology

Wild-type and CCL2 overexpressing mice were either untreated or infected with S. pneumoniae and then killed at various time points postinfection. Subsequently, lungs were inflated in situ with a prewarmed solution of Tissue-Tek (Sakura) kept at 37°C. Thereafter, the lungs were carefully removed and immersed in PBS-buffered formaldehyde solution (4.5%, pH 7.0) for at least 24 h fixation at room temperature. Subsequently, lungs were paraffin-embedded, and lung sections of 5 μm were stained with H&E and Elastica-van-Gieson and examined histopathologically using a Zeiss Axiosvert 200 M microscope.
For the quantification of bronchiolitis obliterans, total areas of two sections of the completely embedded lung specimen of either CCL2 overexpressing or wild-type mice infected with *S. pneumoniae* for 7 days were calculated by planimetry. Subsequently, the number of completely effaced terminal bronchioles was counted and calculated as mesenchymal proliferation per square centimeter of lung tissue.

**ELISA**

Proinflammatory cytokine release in BAL fluids of untreated or *S. pneumoniae*-infected CCL2 overexpressing mice and wild-type mice was determined using commercially available ELISA (R&D Systems and Bender MedSystems).

**Statistics**

All data are given as mean ± SD. Differences between controls and respective treatment groups over time were analyzed by ANOVA followed by post hoc Dunnett test. Significant differences between groups were analyzed by Levene’s test for equality of variances followed by Student’s *t* test using SPSS for Windows software package. Survival curves were compared by log-rank test. Statistically significant differences between various treatment groups were assumed when *p* values were <0.05.

**Results**

Human CCL2 overexpression in type II alveolar epithelial cells elicits increased lung and alveolar mononuclear phagocyte subset accumulations

Initial experiments were performed to characterize the mononuclear phagocyte subset accumulations in the lungs of uninfected wild-type and CCL2 overexpressing mice. As shown in Fig. 1A,

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**FIGURE 1.** Effect of CCL2 overexpression on lung mononuclear phagocyte subset accumulations. A, Analysis of human and murine CCL2 protein levels in BAL fluids of untreated CCL2 overexpressing and wild-type mice. B, Pappenheim-stained cytospin preparations of BAL fluid cellular constituents of untreated wild-type and CCL2 overexpressing mice. Note the abundance of monocytic cells in BAL fluids of CCL2 overexpressing mice exhibiting considerably variable nuclear shapes. C, Representative FACS analysis of mononuclear phagocytes collected by BAL from wild-type mice (*left plots*) and CCL2 overexpressing mice (*right plots*). Mononuclear phagocytes were gated according to their FSC-A vs SSC-A characteristics (*upper*), thereby excluding cellular debris and lymphocytes, as well as according to their F4/80 vs SSC-A characteristics (*middle*). Staining of the cells with anti-CD11b and anti-CD11c mAbs identified one major CD11b<sup>+</sup>, CD11c<sup>+</sup> mononuclear phagocyte population (P) in wild-type mice (P1), and three mononuclear phagocyte subsets (P2, P3, and P4) in CCL2 overexpressing mice. D, Cells for populations P1 and P2 in C were identified as alveolar macrophages, according to their MHC-II<sup>+</sup>, CD86<sup>+</sup>, CD49d<sup>+</sup> phenotype, whereas cells in P3 and P4 subsets were characterized as monocyte-derived myeloid DC (MHC-II<sup>high</sup>, CD86<sup>+</sup>, CD49d<sup>+</sup>, Gr-1<sup>+</sup>) and alveolar monocytic cells (MHC-II<sup>low</sup>, CD86<sup>+</sup>, CD49d<sup>+</sup>, Gr-1<sup>-</sup>), respectively. Open histogram, Cell surface Ag expression, as indicated. Gray-filled histogram, Negative control. E and F, Quantification of mononuclear phagocyte subsets in BAL fluids (*E*) and lung parenchymal tissue (*F*) of wild-type mice and CCL2 overexpressing mice, using the immunophenotypic profiling outlined in C and D. Data are mean ± SD of seven mice per group for A, E, and F. *p* < 0.05; **p** < 0.01 compared with wild-type.
untreated CCL2 overexpressing mice demonstrated strongly increased BAL fluid levels of human CCL2 protein, whereas murine CCL2 protein was not detectable in BAL fluids of uninfected mice of either treatment group, thus confirming and extending previous reports (4). Examination of Pappenheim-stained cytopsins of BAL fluid cellular constituents of the two treatment groups showed that alveolar macrophages were the main cellular constituent in BAL fluids of wild-type mice, while BAL fluids collected from CCL2 overexpressing mice demonstrated strongly expanded lung and alveolar macrophage and 4-fold expanded lung monocytic cell/DC pool in their lung parenchymal tissue compared with wild-type mice (Fig. 1E). In addition, CCL2 overexpressing mice exhibited an ~3-fold expanded lung macrophage and 4-fold expanded lung monocytic cell/DC pool in their lung parenchymal tissue compared with wild-type mice, as shown in Fig. 1F.

CCL2 overexpressing mice show an improved bacterial clearance and increased survival to infection with S. pneumoniae

Because untreated CCL2 overexpressing mice, as opposed to wild-type mice, demonstrated significantly expanded lung and alveolar mononuclear phagocyte subset accumulations, we hypothesized that these mice would demonstrate an improved innate host defense to challenge with S. pneumoniae. CCL2 overexpressing mice infected with S. pneumoniae showed an improved pneumococcal clearance compared with wild-type mice with significant differences noted by day 3 postinfection (Fig. 2A). Importantly, this improved pneumococcal clearance observed in CCL2 overexpressing mice was associated with a significantly increased survival. As shown in Fig. 2B, ~80% of CCL2 overexpressing mice survived by day 5 postinfection, as opposed to ~35% survival rate in the wild-type mice. Microscopic examination of lung tissue sections collected from uninfected and S. pneumoniae-infected wild-type mice and CCL2 overexpressing mice demonstrated a normal lung architecture with regular bronchiolar and alveolar structures in uninfected mice of either treatment group, but established purulent bronchiolitis and alveolitis in S. pneumoniae-infected wild-type mice at day 3 postinfection that was not detected in the CCL2 overexpressing mice (Fig. 2C).

Proinflammatory cytokine responses and neutrophilic alveolitis in CCL2 overexpressing mice as compared with wild-type mice infected with S. pneumoniae

Proinflammatory cytokines and chemokines critically regulate inflammatory leukocytic responses, thereby contributing to the lung innate host defense against inhaled bacterial pathogens. As shown in Fig. 3, uninfected CCL2 overexpressing mice demonstrated similar baseline BAL fluid levels of TNF-α and neutrophil chemotactic MIP-2 and KC as observed in wild-type mice. However, infection of CCL2 overexpressing and wild-type mice with S. pneumoniae significantly increased BAL fluid cytokine levels peaking by day 1 postinfection and rapidly declining thereafter, with no significant differences noted between groups (Fig. 3, A–C). The developing neutrophilic alveolitis observed in CCL2 overexpressing mice ranged in the same order of magnitude as observed in wild-type mice with significant increases observed between day...
CCL2 overexpressing mice respond with drastically increased BAL fluid CCL2 levels and inflammatory mononuclear phagocyte subset accumulations to S. pneumoniae challenge.

We also analyzed human and murine BAL fluid CCL2 protein levels as well as murine CCL7 and CCL12 BAL fluid levels in mice challenged with S. pneumoniae. Human CCL2 protein contents in BAL fluids of CCL2 overexpressing mice infected with S. pneumoniae were significantly higher compared with uninfected transgenic mice with peak levels observed by day 2 postinfection (Fig. 4A). In contrast, murine CCL2 protein levels in BAL fluids of both wild-type and CCL2 overexpressing mice, although being significantly increased compared with uninfected control mice ranged at much lower levels peaking by days 2 and 3 without significant differences noted between groups (Fig. 4B). Lowest CC chemokine levels in BAL fluids of untreated or S. pneumoniae-infected CCL2 overexpressing and wild-type mice were observed for murine chemokines CCL7 and CCL12, with no significant differences noted between groups (Fig. 4, C and D).

We next performed FACS analysis of BAL fluid cellular constituents collected from the lungs of S. pneumoniae-infected CCL2 overexpressing and wild-type mice. Wild-type mice infected with S. pneumoniae showed two major mononuclear phagocyte subsets within the alveolar air space peaking by day 7 postinfection (Fig. 5, A–D). These two populations (P1 and P2 in Fig. 5, C and D) represented resident alveolar macrophages (Fig. 5, C and D, P1), as identified by their F4/80$^{\text{high}}$, CD11c$^{\text{high}}$, CD11b$^{\text{neg}}$, MHC-II$^{\text{neg}}$, CD86$^{\text{neg}}$, CD49d$^{\text{neg}}$ cell surface Ag expression profile, consistent with the mononuclear phagocytes detected under baseline conditions.

1 up until day 3, with a slight but significant increase in the numbers of recruited neutrophils noted in CCL2 overexpressing mice on day 2 postinfection (Fig. 3D). These data demonstrate that human CCL2 transgene expression in mice did not induce baseline release of proinflammatory cytokines in the bronchoalveolar compartment, nor did it affect the release of proinflammatory cytokines or the neutrophilic alveolitis developing in response to pneumococcal challenge.

**FIGURE 3.** Proinflammatory cytokine responses and neutrophilic alveolitis in CCL2 overexpressing compared with wild-type mice infected with S. pneumoniae. Wild-type mice ([]) and CCL2 overexpressing mice (■) were infected with S. pneumoniae for various time points. Subsequently, mice were subjected to BAL, and determination of BAL fluid cytokine profiles with TNF-α (A), MIP-2 (B), and KC (C), or developing neutrophilic alveolitis (D), as indicated. Data are mean ± SD of eight mice per group and time point. +, p < 0.05; ++, p < 0.01; +++, p < 0.001 increase compared with the respective control values. *, p < 0.05 increase compared with wild-type mice.

**FIGURE 4.** Release of monocyte chemoattractants in CCL2 overexpressing and wild-type mice infected with S. pneumoniae. CCL2 overexpressing mice and wild-type mice were either uninfected (d0) or were infected with S. pneumoniae for various time points. Subsequently, mice were subjected to BAL and determination of BAL fluid CCL2, CCL7, and CCL12 chemokine levels. A. Human CCL2 protein in BAL fluids of CCL2 overexpressing mice. B–D. Murine CCL2, CCL7, and CCL12 protein in BAL fluids of wild-type mice ([]) and CCL2 overexpressing mice (■), as indicated. Data shown are mean ± SD of eight mice per group and time point. +, p < 0.05; ++, p < 0.01; +++, p < 0.001 increase compared with respective control (d0) time points.
The second population of exudate mononuclear phagocytes showed a monocytic morphology in Pap- penheim-stained cytospins (data not shown) and demonstrated a differential CD11chigh and CD11b high Ag expression profile, but were consistently found to be MHC-II neg, in conjunction with CD86low, CD49dpos, and Gr-1 neg, thus representing inflammatory recruited monocyte-derived exudate macrophages (Fig. 5, C and D). FACS profiling of mononuclear phagocyte subsets contained in BAL fluids of S. pneumoniae-infected CCL2 overexpressing mice allowed us to again discriminate three populations of mononuclear phagocytes (Fig. 5, E–H, P1–P3), representing alveolar macrophages (P1), alveolar monocyte-derived myeloid DC (P2), and inflammatory monocytes (Fig. 5, G and H, P3) characterized by their CD11chigh but CD11cneg/low, MHC-II neg/low, CD86low, CD49dpos, and Gr-1neg/pos cell surface Ag expression profile (Fig. 5, G and H, P3). Based on this immunophenotypic analysis, quantification of mononuclear phagocyte subsets showed that wild-type mice responded with a drastic and nearly complete depletion of the resident alveolar macrophage pool by days 1 and 2 postinfection, resembling most recently made observations with wild-type mice.
the purified pneumococcal cytolysin pneumolysin (3). This drop in alveolar macrophages noted in wild-type mice was followed by an expansion of alveolar macrophages by days 7–14 of infection (Fig. 5I). In striking contrast, CCL2 overexpressing mice showed much higher numbers of alveolar macrophages from day 2 to 7 of infection with \textit{S. pneumoniae} (Fig. 5I). In addition, CCL2 overexpressing mice showed a significantly increased number of elicited mononuclear phagocyte subsets in their lungs upon infection with \textit{S. pneumoniae} for either 3 or 7 days in the absence or presence of MC21, as indicated. Data shown are mean ± SD of eight mice per group and time point. In F, **, p < 0.01 compared with day 3 or 7 \textit{S. pneumoniae} only infected CCL2 overexpressing mice. +, p < 0.05; ++, p < 0.01 compared with uninfected mice (day 0).

CCL2 overexpressing mice respond to challenge with \textit{S. pneumoniae} with increased bronchiolitis obliterans

Fibroproliferative responses are clinical complications frequently developing in the late phase of acute lung injury, as observed in severe pneumonia leading to acute respiratory distress syndrome (ARDS) (13–15). Examination of lung tissue sections from \textit{S. pneumoniae}-infected mice of the two treatment groups revealed that 75% of the CCL2 overexpressing mice as compared with 25% of the wild-type mice developed bronchiolitis obliterans with organizing pneumonia by day 7 postinfection (Fig. 6A, left). Morphometric analysis of the respective lung tissue sections revealed a significantly increased number of obliterated terminal bronchioles in \textit{S. pneumoniae}-infected CCL2 overexpressing, as compared with wild-type mice, by day 7 postinfection (Fig. 6B). To evaluate the contribution of CCL2-CCR2 mediated cell recruitment processes to such fibroproliferative responses developing in the transgenic mice upon pneumococcal challenge, CCL2 overexpressing mice and wild-type mice received anti-CCR2 Ab MC21, which was recently shown to efficiently block inflammatory CC chemokine interaction with its primary CC chemokine receptor, CCR2 (3, 5, 8). Treatment of CCL2 overexpressing mice with MC21 for up to 7 days completely inhibited the bronchiolitis obliterans formation upon pneumococcal challenge, but at the same time reduced the number of resident and particularly the number of

**FIGURE 6.** Effect of CCR2 blockade on lung histopathology, pneumococcal clearance, and mononuclear phagocyte mobilization in \textit{S. pneumoniae}-infected CCL2 overexpressing mice. A, Lung histopathology of CCL2 overexpressing mice infected with \textit{S. pneumoniae} for 7 days either in the absence (left) or the presence (right) of anti-CCR2 Ab MC21. Bronchiolitis obliterans is indicated by arrows. B, Quantification of bronchiolitis obliterans in lung tissue sections of wild-type mice and CCL2 overexpressing mice at day 7 postinfection. ***, p < 0.02 compared with \textit{S. pneumoniae}-infected wild-type mice. C, Pneumococcal load in whole lung washes of CCL2 overexpressing mice at day 7 postinfection in the absence or presence of MC21 application, as indicated. *, p < 0.05 compared with \textit{S. pneumoniae} only infected CCL2 overexpressing mice. D, Representative FACS dot plot illustrating the effect of MC21 application on the mobilization of mononuclear phagocytes in CCL2 overexpressing mice analyzed at day 7 postchallenge with pneumococcal infection. Total cell number of alveolar macrophages (P1) (E) or alveolar DC (P2) and monocytic cells (P3) (shown as combined total cell number of P2 and P3 cells identified and gated as described in Fig. 5) (F) in CCL2 overexpressing mice challenged with \textit{S. pneumoniae} for 7 days were analyzed. Data shown are mean ± SD of eight mice per group and time point. In F, **, p < 0.01 compared with day 3 or 7 \textit{S. pneumoniae} only infected CCL2 overexpressing mice. *, p < 0.05; ++, p < 0.01 compared with uninfected mice (day 0).
exudate mononuclear phagocyte subsets within the alveolar compartment. The reduction ultimately leads to progressive pneumococcal pneumonia, characterized by an impaired resolution/repair process with development of the classical features of lobar pneumonia (Fig. 6A, right) together with an impaired pneumococcal clearance (Fig. 6C–F). Similarly, disruption of the CCL2-CCR2 axis in wild-type mice by application of MC21 for 7 days also drastically perturbed both the resolution/repair and the pathogen elimination process subsequent to pneumococcal challenge, together with significantly reduced numbers of newly elicited CD11b<sup>pos</sup>/CD11c<sup>pos</sup> macrophages (Fig. 7A, right, and B–E).

**Discussion**

In the current study, we tested the hypothesis that an increased CCL2-dependent lung mononuclear phagocyte mobilization would improve the lung innate host defense to infection with *S. pneumoniae*. We found that CCL2 overexpressing mice exhibited qualitative and quantitative differences in baseline and inflammatory mononuclear phagocyte subset mobilization upon pneumococcal challenge compared with wild-type mice. Although CCL2 overexpressing mice showed an improved pneumococcal clearance and increased survival compared with wild-type mice, they responded with bronchiolitis obliterans to infection with *S. pneumoniae*. Experiments to abrogate the bronchiolitis obliterans by interfering with the CCL2-CCR2 axis induced a progressive pneumococcal pneumonia in the CCL2 overexpressing mice but also in wild-type mice upon treatment with MC21, which was characterized overall by a significantly impaired mononuclear phagocyte mobilization, a reduced pathogen elimination, and an impaired resolution/repair process. These data establish an important role of the CCL2-CCR2 axis in the regulation of both lung innate host defense mechanisms and fibroproliferative responses to bacterial infections.

Previous reports demonstrated that human CCL2 overexpression in type II alveolar epithelial cells elicited a substantial recruitment of monocytic cells both into the lung parenchymal and alveolar compartment of mice in the absence of lung inflammation (4). In the past few years, a detailed phenotypic analysis of murine peripheral blood monocytes has identified two principal subsets, represented by Gr-1<sup>pos</sup> and Gr-1<sup>neg</sup> monocytes. Both subsets are highly CD11b<sup>pos</sup>/CD11c<sup>pos</sup> macrophages (Fig. 7A, right, and B–E).
allowed us to differentiate elicited monocyte cells into monocyte-derived myeloid DC, according to their CD11b, CD11c, MHC-II, and CD86 expression profile, and newly recruited alveolar monocytes, according to their CD11b<sup>pos</sup> and Gr-1<sup>pos</sup> but CD11c<sup>low</sup> and MHC-II<sup>high</sup> Ag expression profile. In contrast, inflammatory exudate mononuclear phagocytes recovered from the lungs of wild-type mice were found to express both CD11b and CD11c, but consistently lacked MHC-II and Gr-1 Ag expression, consistent with an inflammatory exudate macrophage phenotype (19). Interestingly, inflammatory mononuclear phagocyte subset recruitment observed in both wild-type and CCL2 transgenic mice by days 3 and 7 postinfection was nearly completely abrogated by application of anti-CCR2 Ab MC21, whereas the number of alveolar macrophages was less affected, despite an initial drop observed by day 3 postinfection, particularly in the wild-type mice. These data suggest that both CCR2-dependent and –independent processes differentially affect pool sizes of mononuclear phagocyte subsets in response to pneumococcal challenge.

CCL2 overexpressing mice infected with <i>S. pneumoniae</i> were largely protected from severe pneumococcal pneumonia, as opposed to wild-type mice exhibiting an increased mortality as early as day 3 postinfection, where ~50% of the mice succumbed to pneumococcal infection. Notably, the initially developing proinflammatory mediator release and the neutrophilic alveolitis observed by day 3 postinfection were similar in both treatment groups, and thus most likely did not contribute to the observed differences in survival rates between groups. However, major differences between groups were observed with respect to the number of alveolar macrophages and newly elicited mononuclear phagocytes particularly at days 2 and 3 postinfection, being significantly elevated in the transgenic mice as opposed to wild-type mice. Mechanistically, a recent report from our group (3) strongly supports the concept that virulence factors released by <i>S. pneumoniae</i>, particularly pneumolysin, are causally involved in the observed drop in macrophage numbers noted in the wild-type mice upon pneumococcal challenge, whereas CCL2 overexpressing mice were more protected from inflammatory macrophage depletion upon pneumococcal infection, most likely due to their constitutively expanded pool of resident alveolar macrophages. In this context, a recent report suggested that experimental depletion of alveolar macrophages by liposomol clodronate did not affect the early bacterial pathogen elimination process in mice challenged with <i>S. pneumoniae</i> (1). However, the data of the current study demonstrate that first, pneumococcal infection itself is sufficient to transiently deplete the pool of resident alveolar macrophages, which may well explain the significant delay in the bacterial clearance noted in wild-type mice despite the presence of alveolar accumulating neutrophils, thus being consistent with previous reports (20). Second, CCL2 overexpressing mice infected with <i>S. pneumoniae</i> demonstrated a significantly lower drop in macrophage numbers compared with wild-type mice, and at the same time showed an accelerated expansion of the pool of alveolar macrophages together with a significantly increased bacterial pathogen elimination by day 3 postinfection compared with wild-type mice. Thus, the presented data support the view that an increased inflammatory macrophage pool size as observed in CCL2 overexpressing mice may well improve the innate immune response of the lung to infection with <i>S. pneumoniae</i>.

In a recently published study, Dessing et al. (21) reported that CCL2 did not contribute to protective immunity against pneumococcal infection. However, several important differences between that report and our study need to be considered. That report used an intranasal application route to infect CCL2-deficient mice on a C57BL/6 background with serotype 3 <i>S. pneumoniae</i> known to rapidly progress to invasive pneumococcal disease. In contrast, our study infected mice via intratracheal application of capsular group 19 <i>S. pneumoniae</i> known to primarily cause a classical lobar pneumonia in mice. Moreover, in the current study, we used transgenic mice (on a BALB/c background) with a lung- and cell-type-specific overexpression (rather than global deletion) of CCL2, resulting in an increased baseline and particularly inflammatory mononuclear phagocyte recruitment upon challenge with <i>S. pneumoniae</i>. Of note, in the study of Dessing et al. (21), alternatively acting CC chemokines other than CCL2 such as CCL7 and/or CCL12 as part of a redundant chemokine network may have possibly compensated the CCL2 deficiency, but corresponding data have not been reported. At least in our study, murine CCL7 and CCL12 chemokines in BAL fluids of <i>S. pneumoniae</i>-infected CCL2 overexpressing and wild-type mice ranged at much lower levels compared with murine CCL2 and particularly human CCL2, thus strongly arguing against, though not fully excluding, a role for these chemokines in protective immunity observed in <i>S. pneumoniae</i>-infected CCL2 overexpressing mice. Future experiments are required to further investigate the roles of CCL7 and CCL12 in the lung host defense against pneumococcal challenge.

We most recently demonstrated that monocyte-derived lung myeloid DC contribute to pathogen uptake in the acute phase of mycobacterial infections and also provide protection to intracellular pathogens such as <i>Listeria monocytogenes</i> (22, 23). However, the role of inflammatory elicited mononuclear cells and DC in the lung host defense to pneumococcal infection has not been examined so far. It is conceivable that increased lung monocyte/DC numbers noted in human CCL2 overexpressing mice also contributed to innate resistance to pneumococcal challenge, in addition to the aforementioned established role of macrophages in this process. Notably, human CCL2 is known to bind to murine CCR2 with a similar affinity as murine CCL2 and is also able to induce calcium fluxes and murine monocyte/macrophage chemokinesis in vitro, albeit with a lower potency than its murine counterpart (4, 24, 25). Thus, we cannot exclude that overexpression of murine instead of human CCL2 in mice subsequently infected with <i>S. pneumoniae</i> would result in stronger mononuclear phagocyte migratory responses. Importantly, MC21 treatment of <i>S. pneumoniae</i>-infected mice significantly reduced the inflammatory mononuclear phagocyte subset mobilization both in human CCL2 overexpressing and wild-type mice, while leaving the number of alveolar macrophages largely unaffected. At the same time, we observed a progressively developing pneumococcal pneumonia characterized by a significantly delayed pneumococcal clearance and a severely impaired lung resolution/repair process, which may support a role of elicited mononuclear phagocytes in lung innate host defense to pneumococcal challenge. However, we currently cannot distinguish the roles that individual mononuclear phagocyte subsets (inflammatory monocytes vs monocyte-derived DC) play in protective immunity to pneumococcal infection. In contrast, the presented data suggest an interdependence between resolution/repair and pathogen elimination processes because interruption of the CCL2-CCR2 axis to block the mononuclear phagocyte mobilization toward infected lungs led to an increased accumulation of necrotic neutrophils within distal airspaces together with a strongly impaired bacterial pathogen elimination. Given that the baseline turnover of lung mononuclear phagocytes is dramatically accelerated in response to inflammatory challenge (26), the current data further support the critical role of newly elicited mononuclear phagocytes in the lung innate host defense to bacterial infections.

CCL2 overexpressing mice infected with <i>S. pneumoniae</i> developed bronchiolitis obliterans organizing pneumonia with peak histopathological manifestations observed by day 7 postinfection and...
resolving thereafter. Bronchiolitis obliterans is an unspecific fibroproliferative response of the lungs to various inflammatory triggers, such as respiratory syncytial virus infection, lung transplantation, or bleomycin challenge (27). Current concepts regarding the pathophysiology of lung fibroproliferative responses suggest the recruitment of fibroelastic precursor cells (fibrocytes) as an important event in the developing fibrogenic cascades (28, 29). Although it is conceivable that such fibrocyte recruitment is also involved in bronchiolitis obliterans observed in the lungs of S. pneumoniae-infected CCL2 overexpressing mice, this aspect awaits further investigation. However, since murine fibrocytes are known to express CCR2 and are recruited in a CCR2-dependent manner toward the FITC-injured lungs of wild-type mice but not CCR2-deficient mice, and have also been shown to respond to CCL2 treatment with increased collagen secretion (30), human CCL2 overexpression in the lungs of mice might also elicit an increased CCR2-dependent fibrocyte migration upon pneumococcal infection. Importantly, uninfected CCL2 overexpressing mice did not spontaneously develop bronchiolitis obliterans, indicating that elevated CCL2 levels within the bronchoalveolar compartment were not sufficient to elicit fibroproliferation in these mice. Rather, inflammatory cell recruitment and activation elicited by pneumococcal infection and/or pneumococci-derived pathogen-associated molecular patterns in the presence of increased CCL2 levels was required to elicit the fibroproliferative response in CCL2 overexpressing mice. Although MC21 treatment completely inhibited the bronchiolitis obliterans with organizing pneumonia formation in S. pneumoniae-infected CCL2 overexpressing mice, as did anti-CCL2 Ab administration in a recent tracheal transplant model (31), these intervention strategies do not allow selective discrimination between fibrogenic effects possibly mediated by CCR2R-positive lung mononuclear phagocyte subsets and/or corecruited CCR2-positive fibrocytes. Interestingly, most recent reports demonstrated that mesenchymal precursors of a monocyte/macrophage lineage contributed to hypoxia-induced pulmonary vascular remodelling and possibly to ischemic cardiomyopathy in mice, thus possibly linking the generation of fibrocytes to hemopoietic precursors of a monocyte/macrophage lineage (32, 33). Recent observations from our group demonstrated that highly elevated CCL2 levels in BAL fluids of critically ill patients with septic ARDS were associated with the severity of respiratory failure (15). Because septic ARDS patients are at considerably increased risk of developing fibrotic responses in the late phase of the disease, it will be important to evaluate whether CCL2-triggered mechanisms outlined in the current study may also apply to patients with septic ARDS, where increased CCL2 levels and sustained inflammatory activation coincide within the same lung compartment. Collectively, the current study demonstrates that CCL2 overexpressing mice exhibiting an increased number of mononuclear phagocytes in their lungs were more resistant to challenge with S. pneumoniae than wild-type mice, but showed fibroproliferative responses later during pneumococcal lung infection. Strategies to interrupt the CCL2-CCR2 axis in these mice abrogated the fibroproliferative response, but severely attenuated the resolution/repair process and pathogen elimination in S. pneumoniae-infected mice. The presented data provide further insight into the role of the CCL2-CCR2 axis both in the regulation of bacterial pathogen elimination and resolution-repair processes as well as pathogen-induced lung fibroproliferative responses.

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Disclosures

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References


