Funktion mononukleärer Phagozyten in der Pathogenese der *Streptococcus pneumoniae*-induzierten Pneumonie der Maus

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für Ole

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Abkürzungsverzeichnis

AM	Alveolarmakrophage
ARDS	Acute respiratory distress syndrome
BAL	Bronchoalveoläre Lavage
CAP	Community-aquired pneumonia
CbpA	Choline-binding protein A
CCL2	CC Chemokin Ligand 2
CCR2	CC Chemokin Rezeptor 2
CD	Cluster of differentiation
CMP	Common myeloid progenitor
CRM_{197}	Cross-reactive material 197
CXCR2	CXC Chemokin Rezeptor 2
DC	Dendritische Zelle
FACS	Fluorescence-activated cell sorting
Flt3L	Fms-like tyrosine kinase-3 ligand
FSC	Forward scatter
GM-CSF	Granulocyte/macrophage-colony stimulating factor
GPCR	G-protein coupled receptor
IL-8	Interleukin-8
KC	Keratinocyte-derived cytokine
kDa	Kilodalton
KO	Knockout
LPS	Lipopolysaccharid
MCP-1	Monocyte chemoattractant protein-1
MDP	Macrophage/DC progenitor
MHCII	Major histocompatibility complex II
MIP-2	Macrophage inflammatory protein-2
MPS	Mononukleär-phagozytäres System
PAMP	Pathogen-associated molecular pattern
PGN	Peptidoglykan
PI3Kγ	Phosphatidylinositol 3,4,5-triphosphate kinase γ
PIP	Phosphatidylinositol
PKB	Proteinkinase B
PLY	Pneumolysin

PMN	Polymorphonuclear neutrophil
PRR	Pattern recognition receptor
PspA	Pneumococcal surface protein A
SP-C	Surfactant Protein C
SSC	Side scatter
tg	transgenic
TLR	Toll-like receptor
7-TMS	Seven-transmembrane-spanning
TNFα	Tumor Necrosis Factor α
WT	Wildtyp

Zusammenfassung

Infektionen der Lunge mit *Streptococcus pneumoniae* stellen weltweit nach wie vor ein großes medizinisches Problem mit hoher Morbidität und Letalität dar. Die stetige Zunahme von Resistenzen gegenüber den wichtigsten Antibiotika erschwert die Therapie der Pneumonie zunehmend und macht es zwingend erforderlich, alternative Behandlungsmethoden zu finden. Dieses setzt jedoch ein besseres Verständnis der Wirts-Pathogen-Interaktionen in der Lunge sowie der ihr zugrunde liegenden Pathomechanismen voraus. Eine der wichtigsten Abwehrzellen der Lunge gegenüber inhalierten bakteriellen Erregern ist der residente Alveolarmakrophage, welcher zum mononukleär-phagozytären System (MPS) gehört. In der vorliegenden Arbeit wurde die Bedeutung des mononukleär-phagozytären Systems in der Pathogenese der *Streptococcus pneumoniae*-induzierten Pneumonie der Maus untersucht.

Neuere Untersuchungen haben gezeigt, dass der Phospholipidkinase Phosphatidylinositol 3-Kinase γ (PI3Kγ) eine zentrale Rolle in der entzündlichen Leukozytenrekrutierung zukommt. Die Untersuchungen der vorliegenden Arbeit zur Bedeutung der PI3Kγ in der *S. pneumoniae*-induzierten Pneumonie haben gezeigt, dass die Deletion oder Inhibition der PI3Kγ zu einer massiven Reduktion der *S. pneumoniae*-induzierten Rekrutierung mononukleärer Phagozyten in das alveoläre Kompartiment führte mit der Konsequenz, dass sowohl PI3Kγ defiziente als auch mit oral verfügbaren Inhibitoren gegen PI3Kγ vorbehandelte Wildtyp-Mäuse eine signifikant reduzierte Infektabwehr hatten und eine gestörte Reparaturphase im späteren Verlauf der Pneumokokken-Pneumonie aufwiesen. Demgegenüber konnten wir zeigen, dass Mäuse mit einer konstitutiven, lungenspezifischen Überexpression des Monozyten-rekrutierenden Chemokins CCL2 eine erheblich beschleunigte und verstärkte Erregerelimination, als auch eine signifikant reduzierte Letalität und somit verbesserte Wirtsabwehr gegenüber *S. pneumoniae* aufwiesen. Darüber hinaus konnte gezeigt werden, dass CCL2 transgene Mäuse nach Infektion mit *S. pneumoniae* eine erheblich verbesserte Heilungs- und Reparaturphase im Verlauf der Pneumokokken-Pneumonie aufwiesen, wenngleich sich in der Spätphase der Infektion in den Lungen CCL2 transgener Mäuse fibroproliferative Umbauprozesse nachweisen liessen. Weiterführende Untersuchungen zur Charakterisierung der Funktion individueller Phagozyten-Subpopulationen (Subsets) in der Pathogenese der Pneumokokken-Pneumonie zeigten, dass eine transient verstärkte Akkumulation

myeloider dendritischer Zellen in den Lungen von Mäusen durch Vorbehandlung mit dem hämatopoetischen Wachstumsfaktor Fms-like tyrosine kinase 3 ligand (Flt3L) zu einer massiven Verschlechterung der Wirtsabwehr gegenüber *S. pneumoniae* führte, charakterisiert durch eine gesteigerte Letalität, eine schlechtere Keimelimination sowie eine massiv gesteigerte Vaskulitis und erhöhte Lungenpermeabilität. Die Daten dieser Untersuchungen legen demzufolge den Schluss nahe, dass die globale Attenuierung der mononukleären Phagozytenrekrutierung in die Lunge zu einer Verschlechterung der Erregerelimination und zu einer massiven Störung der Reparaturphase im Verlauf der Pneumokokken-Pneumonie führt. Im Gegensatz dazu führt eine konstitutiv gesteigerte mononukleäre Phagozytenakkumulation in der Lunge zu einer verbesserten Infektabwehr sowie einer deutlich verbesserten Reparaturphase im Verlauf der Pneumokokken-Pneumonie. Eine verstärkte Akkumulation myeloider dendritischer Zellen im Lungenparenchym von Mäusen erbringt demgegenüber aber wahrscheinlich keinen Vorteil bei der Abwehr der *S. pneumoniae*-induzierten Pneumonie und kommt somit für eine therapeutische Intervention nicht infrage.

Während im Modell der konstitutiven lungenspezifischen Überexpression von CCL2 ein klarer Vorteil für den Wirt hinsichtlich Schweregrad und Verlauf der *S. pneumoniae*-induzierten Lungeninfektion dargestellt werden konnte, zeigten weiterführende Untersuchungen in *S. pneumoniae*-infizierten CCL2-defizienten Mäusen, dass die Deletion von CCL2 zu einer erheblichen Beeinträchtigung der protektiven Immunabwehr der Lunge gegenüber *S. pneumoniae* führte, gekennzeichnet durch eine sich rasch entwickelnde Bakteriämie, eine signifikant reduzierte inflammatorische Exsudatmakrophagenmobilisierung assoziiert mit einer dramatisch aggravierten Histopathologie sowie einer signifikant erhöhten Letalität. Der Befund, dass in CCL2 defizienten Mäusen eine Bakteriämie zu beobachten war, obwohl diese Mäuse mit einem nicht invasiv wachsenden Pneumokokken-Stamm infiziert wurden, lässt den Schluss zu, dass entzündlich rekrutierte Exsudatmakrophagen eine „Barriere“-Funktion in der Lunge übernehmen und somit massgeblich zur Infektabwehr der Lunge gegenüber bestimmten *S. pneumoniae*-Serotypen beitragen.

Zusammengefasst unterstützen die in der vorliegenden Arbeit präsentierten Daten das Konzept, dass die im Verlauf pulmonaler bakterieller Infektionen in das Lungenparenchym und alveoläre Kompartiment rekrutierten inflammatorischen

Monozyten und hieraus differenzierende Exsudatmakrophagen eine zentrale Rolle im Prozess der Heilungs- und Reparaturphase spielen, indem diese Zellen im Verlauf einer Pneumokokken-Pneumonie an der Beseitigung der entstehenden konsolidierten Infiltrate beteiligt sind. Die Attenuierung bzw. Blockade der inflammatorischen Monozytenrekrutierung durch Deletion von CCL2, funktionsblockierenden Antikörpern gegen CCR2 sowie Inhibition der ‚downstream' von CCR2 agierenden PI3Kγ führt zu einer massiven Störung der Exsudatmakrophagenmobilisierung, welches wiederum zu einer massiven Störung der Reparaturphase und der Erregerelimination im Verlauf der Pneumokokken-Pneumonie führt. Hieraus lässt sich ableiten, dass es in der pulmonalen Wirtsabwehr inhalierter bakterieller Erreger einen zentralen funktionellen Zusammenhang zwischen einer effizienten Reparaturphase und der Beseitigung der Erreger aus dem Lungenkompartiment im Verlauf der Pneumonie gibt.

Schlagwörter: *Streptococcus pneumoniae*, Pneumonie, Lunge

Abstract

Streptococcus pneumoniae is the most prevalent pathogen causing community-acquired pneumonia (CAP) worldwide. Increasing antibiotic resistance of *S. pneumoniae* requires development of alternative treatment regimens to antagonize pneumococcal infections. In the present thesis, the role and function of mononuclear phagocytes in the pathogenesis of *S. pneumoniae*-induced lung infection in mice was analyzed. In initial experiments, we evaluated the role of the phospholipidkinase Phosphoinositide 3-kinase γ (PI3Kγ), which plays a critical role in inflammatory leukocyte recruitment, in mice challenged with *S. pneumoniae.* We found that PI3Kγ deficiency led to a substantially reduced *S. pneumoniae*-induced exudate macrophage recruitment into the lung, which was accompanied by a significantly impaired pathogen elimination and resolution/repair process subsequent to pneumococcal infection. In contrast, mice with an overexpression of CCL2 in type II alveolar epithelial cells that are characterized by a constitutively increased mononuclear phagocyte subset mobilisation into the alveolar space, responded with an increased bacterial clearance and a significantly improved resolution/repair process together with a significantly increased survival relative to *S. pneumoniae*-infected wild-type mice. To analyze the role for individual mononuclear phagocyte subsets such as dendritic cells (DC) in protective immunity against inhaled bacterial pathogens, we pre-treated mice with the dendritic cell-specific growth factor Fms-like tyrosine kinase-3 ligand (Flt3L) prior to infection of the mice with *S. pneumoniae*. Such increased accumulation of DC in lung parenchymal tissue prior to infection with *S. pneumoniae* led to a significantly decreased bacterial clearance, increased vasculitis and lung permeability together with increased mortality compared to wild-type mice infected with *S. pneumoniae*. Finally, CCL2 KO mice lacking inflammatory exudate macrophage recruitment in response to *S. pneumoniae* infections demonstrated increased bacteraemia and mortality compared to wild-type mice. Thus, the data of the current thesis demonstrate that CCL2-dependent mononuclear phagocyte subset recruitment into the lungs of mice is a critical determinant in the lung host defense against inhaled bacterial pathogens. Evidence is provided to support the concept that exudate macrophages not only serve as barrier cells to prevent non invasive *S. pneumoniae* to escape local immune surveillance and cause bacteraemia in mice (CCL2 KO), but also are critical cellular components for the later

developing resolution/repair process to eliminate consolidated cellular infiltrates from lung parenchymal tissue to reconstitute lung homeostasis. Disturbing the resolution/repair process by either blocking or inhibiting CCL2 dependent exudate macrophage mobilisation into the lungs, as observed in PI3Kγ deficient or inhibitor pre-treated mice, or in CCL2 KO mice, results in a significantly impaired lung resolution/repair process subsequent to pneumococcal lung infection. Thus, an efficient resolution/repair phase is not only important for regaining lung homeostasis but also indirectly affects the pathogen elimination process in the later phase of the disease. Thus, the presented data suggest that therapeutic intervention strategies targeting the amount and kinetics of exudate macrophage recruitment to the lungs may be successful in improving protective immune responses of the lung against inhaled bacterial pathogens.

Keywords: *Streptococcus pneumoniae*, pneumonia, lung

1. Einleitung

1.1 *Streptococcus pneumoniae*

Streptococcus pneumoniae (Pneumokokkus) ist der weltweit häufigste Erreger der gesunden Bevölkerung für ambulant erworbene Pneumonien (community-acquired pneumonia, CAP) [1]. Als kommensales Bakterium besiedelt *S. pneumoniae* die Schleimhäute des oberen Respirationstraktes von 30-50% der gesunden Bevölkerung, ohne Symptome einer Atemwegserkrankung hervorzurufen. Neben der ambulant erworbenen Pneumonie sind Pneumokokken auch kausal an der Entstehung von Nasennebenhöhlenentzündungen (Sinusitis), der Mittelohrentzündung (Otitis media) und der Hirnhautentzündung (Meningitis) beteiligt [2]. *Streptococcus pneumoniae* ist ein bekapseltes Gram-positives Bakterium, dessen primäre Wuchsform diplokokkal (*Diplococcus pneumoniae*) ist; andererseits können Pneumokokken aber auch in langen Ketten als „Strepto“-kokken wachsen. Gegenwärtig sind mehr als 90 verschiedene Serotypen bekannt, welche nach der Immunogenität der bakteriellen Polysaccharidkapsel unterschieden werden können [2, 3]. Die Polysaccharidkapsel der Pneumokokken spielt in der Pathogenese und Virulenz des Erregers eine wichtige Rolle. Versuche von Griffith in den zwanziger Jahren des letzten Jahrhunderts mit bekapselten und unbekapselten Pneumokokken zeigten, dass nur die bekapselten Bakterien in der Lage waren, in Labormäusen eine Infektion hervorzurufen, während die unbekapselten Pneumokokken avirulent waren [4, 5]. Die unter der Polysaccharidkapsel befindliche Zellwand besteht neben Polysacchariden aus Teichon- und Lipoteichonsäurebausteinen. Darüber hinaus trägt die Zellwand von *S. pneumoniae* zellwandassozierte Oberflächenproteine, wie z.B. das pneumokokkale Oberflächenprotein A (pneumococcal surface protein A, PspA) oder aber das Cholin bindende Protein A (choline-binding protein A, CbpA) [6]. Einige dieser Oberflächenproteine, wie z.B. PspA, verhindern aufgrund elektrostatischer Ladungsverteilungen im Protein die Anlagerung von Komplementfaktoren an die Erreger und behindern dadurch sehr effizient die Komplement-abhängige Phagozytose der Erreger durch professionelle Phagozyten. Wiederum andere pneumokokkale Oberflächenproteine ermöglichen es den Erregern, mit Epithelzellen des Wirtes zu interagieren und erleichtern somit die Kolonisation der Atemwegsschleimhäute [3, 7].

Einer der wichtigsten Virulenzfaktoren von *S. pneumoniae* ist das Pneumolysin [8]. Pneumolysin ist ein 53 kDa großes Protein und wird von allen klinischen Pneumokokken-Isolaten produziert und trägt maßgeblich zum invasiven Wachstumsverhalten von Pneumokokken im Wirtsorganismus bei. Pneumolysin liegt als intrazelluläres Zytolysin im Zytoplasma des Erregers vor und wird mittels Autolyse der Bakterienzellen freigesetzt. Nach der Freisetzung des Toxins binden die Toxin-Monomere an Cholesterol der Zellmembran und oligomerisieren dort zu Transmembranporen, was zur Lyse der betroffenen Zellen führt [9]. Neben dieser direkten zytotoxischen Wirkung des Pneumolysins konnte gezeigt werden, dass Pneumolysin als PAMP an den Mustererkennungsrezeptor TLR4 binden kann und proinflammatorische Entzündungsantworten in Makrophagen als Teil der angeborenen Immunabwehr auslöst [10, 11].

1.2 Die Pneumonie

Die ambulant erworbene Lungenentzündung ist in 40-60% aller Fälle auf Infektionen mit *Streptococcus pneumoniae* zurückzuführen. *S. pneumoniae* ist zudem der am häufigsten nachgewiesene Erreger der CAP-assoziierten Bakteriämie [12]. Neben Pneumokokken spielen jedoch in den letzten Jahren auch andere Erreger, wie insbesondere *Staphylococcus aureus*, *Haemophilus influenzae, Klebsiella pneumoniae* sowie *Pseudomonas aeruginosa* im Krankenhaus als Verursacher bakterieller Lungeninfektionen eine wichtige Rolle. Vor allem Kleinkinder und Erwachsene über 60 Jahre erkranken an CAP. Etwa 600.000 Menschen pro Jahr entwickeln in Deutschland eine Pneumonie; von diesen werden ca. 30% im Krankenhaus behandelt [13]. Klinisch äußert sich das Bild einer Pneumonie mit den Symptomen Fieber, Husten, Auswurf, Atemnot und Brustschmerzen. Je nachdem, welcher Bereich der Lunge von der Infektion betroffen ist, spricht man von einer Lobärpneumonie (Lappenpneumonie) oder einer Bronchopneumonie. Im Falle der Lobärpneumonie sind einzelne Lungensegmente bzw. Lungenlappen von der Infektion betroffen, während die Bronchopneumonie eine primär von den großen Atemwegen ausgehende Infektion mit Pneumokokken darstellt, welche sich jedoch im weiteren Verlauf auf distale Lungenareale ausbreiten kann. Besonders schwere Verläufe einer Pneumokokken-Pneumonie gehen mit der Entwicklung von Bakteriämie und Sepsis einher (invasiver Krankheitsverlauf), welches nicht selten

auch die Entwicklung einer septischen Meningitis zur Folge haben kann. Darüber hinaus kommt es bei Pneumonien mit schweren Krankheitsverläufen häufig zur Entwicklung einer respiratorischen Insuffizienz, was in der Regel eine intensivmedizinische Überwachung der betroffenen Patienten erfordert. Die stationär behandelte ambulant erworbene Pneumonie (CAP) geht mit einer Letalität („in-hospital mortality“) von ca. 14 % einher [14].

Aufgrund der zunehmenden Entwicklung von Antibiotikaresistenzen bei CAP-relevanten Keimen, so auch *S. pneumoniae*, ist die Entwicklung neuer Impfstrategien und alternativer Interventionsmaßnahmen zur Therapie von Pneumokokkeninfektionen wichtiger denn je. In der Klinik stehen für die Pneumokokkenimpfung von Kindern und Erwachsenen gegenwärtig zwei zentrale Impfstrategien zu Verfügung: zum einen handelt es sich um eine 23-valente Polysaccharidvakzine (Pneumovax® und Pneumopur®), welche jedoch nur eine primär IgM vermittelte Immunität gegen 23 der >90 gegenwärtig bekannten Pneumokokkenserotypen induziert. Gleichzeitig bietet diese 23-valente Polysaccharidvakzine nur einen 70%igen Schutz vor invasiven Pneumokokkeninfektionen, schützt jedoch nicht vor der Pneumokokken-Pneumonie selbst und ist zudem bei Kleinkindern unter 2 Jahren, wahrscheinlich aufgrund des noch unreifen Immunsystems nicht ausreichend effektiv [15]. Daneben existiert seit kurzem ein 7-valenter Pneumokokken-Konjugatimpfstoff (Prevenar®), in welchem Kapselpolysaccharide der 7 für pädiatrische Pneumonien wichtigsten Pneumokokkenserotypen enthalten sind. In diesem Impfstoff sind die Kapselpolysaccharide an das immunogene Diphterie-Toxoid CRM_{197} (cross-reactive material 197) gekoppelt; dieser 7-valente Konjugatimpfstoff bewirkt nunmehr auch eine suffiziente Immunisierung von Kleinkindern <2 Jahren und trägt somit entscheidend zur Herdenimmunität bei [16]. Andererseits zeichnet sich jedoch bereits nach kurzer Zeit ein ‚Shift’ hin zu Pneumokokkenerkrankungen ab, welche durch nicht von dem Impfstoff abgedeckte Pneumokokken-Serotypen verursacht werden, sodass mit Vakzinierungsstrategien alleine keine ausreichende Senkung der hohen Letalität dieser Erkrankung zu erwarten ist [17].

1.3 Das mononukleär-phagozytäre System (MPS)

Das mononukleär-phagozytäre System (MPS) des Menschen wie auch der Maus stellt die Gesamtheit aller mononukleären Phagozyten der Organsysteme eines Organismus dar. Nach aktuellen Konzepten entwickeln sich die zellulären Bestandteile des MPS aus einer pluripotenten Stammzelle des Knochenmarks, aus der nach einer weiteren Teilung so genannte myeloide Vorläuferzellen (CMP, Abb. 1) hervorgehen [18]. Aus diesen myeloiden Vorläuferzellen entwickeln sich im Knochenmark wiederum Myeloblasten und Makrophagen/DC Progenitorzellen (MDP, Abb. 1). Unter der Wirkung verschiedener Wachstumsfaktoren differenzieren aus den Myeloblasten neutrophile Granulozyten (PMN) und aus den Makrophagen/DC Vorläuferzellen (MDP) die Zellen des mononukleär-phagozytären Systems (Abb. 1) [18-20].

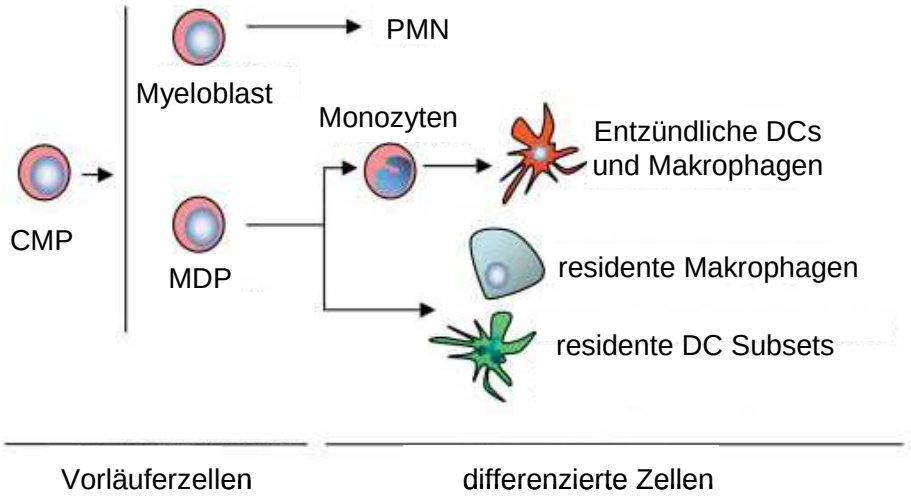


Abb. 1: Ursprung und Differenzierung von mononukleären Phagozyten-Subsets. Die Stammzellen des Knochenmarks (CMP, common myeloid progenitor) differenzieren unter anderem in Myeloblasten und in Makrophagen/DC Vorläuferzellen (MDP, macrophage/DC progenitor). Aus Myeloblasten differenzieren unter Beeinflussung des Granulocyte colony-stimulating factor G-CSF neutrophile Granulozyten (PMN). Aus der MDP Vorläuferzelle differenzieren demgegenüber einerseits Monozyten, sowie residente dendritische Zell-Subsets in den verschiedenen Organsystemen (verändert nach Fogg et al.) [18].

Neuere Untersuchungen haben gezeigt, dass periphere Blutmonozyten der Maus, ähnlich wie beim Menschen, aus zwei verschiedenen Monozyten-Subpopulationen bestehen [21-23]. Beide Subpopulationen peripherer Blutmonozyten der Maus

exprimieren auf ihrer Zelloberfläche CD115, den Rezeptor für den Wachstumsfaktor M-CSF (Macrophage-Colony Stimulating Factor), das β2-Integrin CD11b und das Monozyten/Makrophagen-spezifische Oberflächenantigen F4/80. Demgegenüber differieren die beiden Monozyten-Subsets hinsichtlich ihrer Oberflächenexpression des myeloiden Oberflächenproteins Gr-1 sowie des Chemokinrezeptors CCR2 [19, 20]. Während die sogenannten nicht-inflammatorischen Monozyten des peripheren Blutes $CD11b^{pos}$, $Gr\text{-}1^{neg}$ und $CCR2^{neg}$ sind, zeichnen sich hingegen inflammatorische Monozyten durch ihr $CD11b^{pos}$, $Gr\text{-}1^{pos}$ und $CCR2^{pos}$ Oberflächenantigenexpressionsprofil aus [21, 24, 25]. Nach gegenwärtigem Konzept emigrieren nicht-inflammatorische Monozyten aus dem Blutkreislauf in die verschiedenen Organsysteme, um dort in gewebsständige Makrophagen zu differenzieren. In der Lunge gehen somit aus $Gr\text{-}1^{neg}/CCR2^{neg}$ Blutmonozyten residente Alveolarmakrophagen und myeloide dendritische Zellen des Lungeninterstitiums hervor (Abb. 2A). Demgegenüber werden $Gr\text{-}1^{pos}/CCR2^{pos}$ inflammatorische Blutmonozyten ausschliesslich unter Entzündungsbedingungen in die Lunge rekrutiert. Über die Bedeutung inflammatorisch in die Lunge rekrutierter Monozyten und daraus hervorgehender Exsudatmakrophagen sowie inflammatorisch mobilisierter dendritischer Zellen in der Pathobiologie der akuten Entzündung der Lunge liegen bis dato nur unzureichende Erkenntnisse vor. Abbildung 2B illustriert schematisch die initialen Entzündungskaskaden, welche im alveolären Kompartiment der Lunge nach Infektion mit *S. pneumoniae* induziert werden. Demnach kommt es infolge einer bakteriellen Infektion durch *S. pneumoniae* zur Aktivierung von Alveolarmakrophagen und Alveolarepithelzellen. Die Zellen des Alveolarepithels produzieren als Antwort auf die *S. pneumoniae*-induzierte Freisetzung proinflammatorischer Zytokine durch Alveolarmakrophagen die Neutrophilen-rekrutierenden Chemokine IL-8/MIP-2 bzw. KC (Keratinocyte-derived cytokine), sowie das Monozyten-rekrutierende Chemokin CCL2. Nach Ausbildung eines sich zwischen Kapillarbett der Lunge und Alveolarraum etablierenden Chemokingradienten binden die Chemokine an ihre primären Rezeptoren CXCR2 auf neutrophilen Granulozyten bzw. CCR2 auf inflammatorischen Monozyten [26]. Die Interaktion Neutrophilen-spezifischer Chemokine wie IL-8/MIP-2 mit ihrem Rezeptor CXCR2 auf zirkulierenden neutrophilen Granulozyten sowie die CCL2 Interaktion mit seinem Rezeptor CCR2 auf der Oberfläche inflammatorischer Monozyten induziert konsekutive, direktionale „Migrations-Wellen“ einer frühen

neutrophilen Granulozytenrekrutierung, gefolgt von einer verzögert einsetzenden inflammatorischen Monozytenrekrutierung aus dem Kapillarbett der Lunge durch das Lungeninterstitium in den Alveolarraum, wo diese Zellen per bronchoalveolärer Lavage nachgewiesen werden können [27].

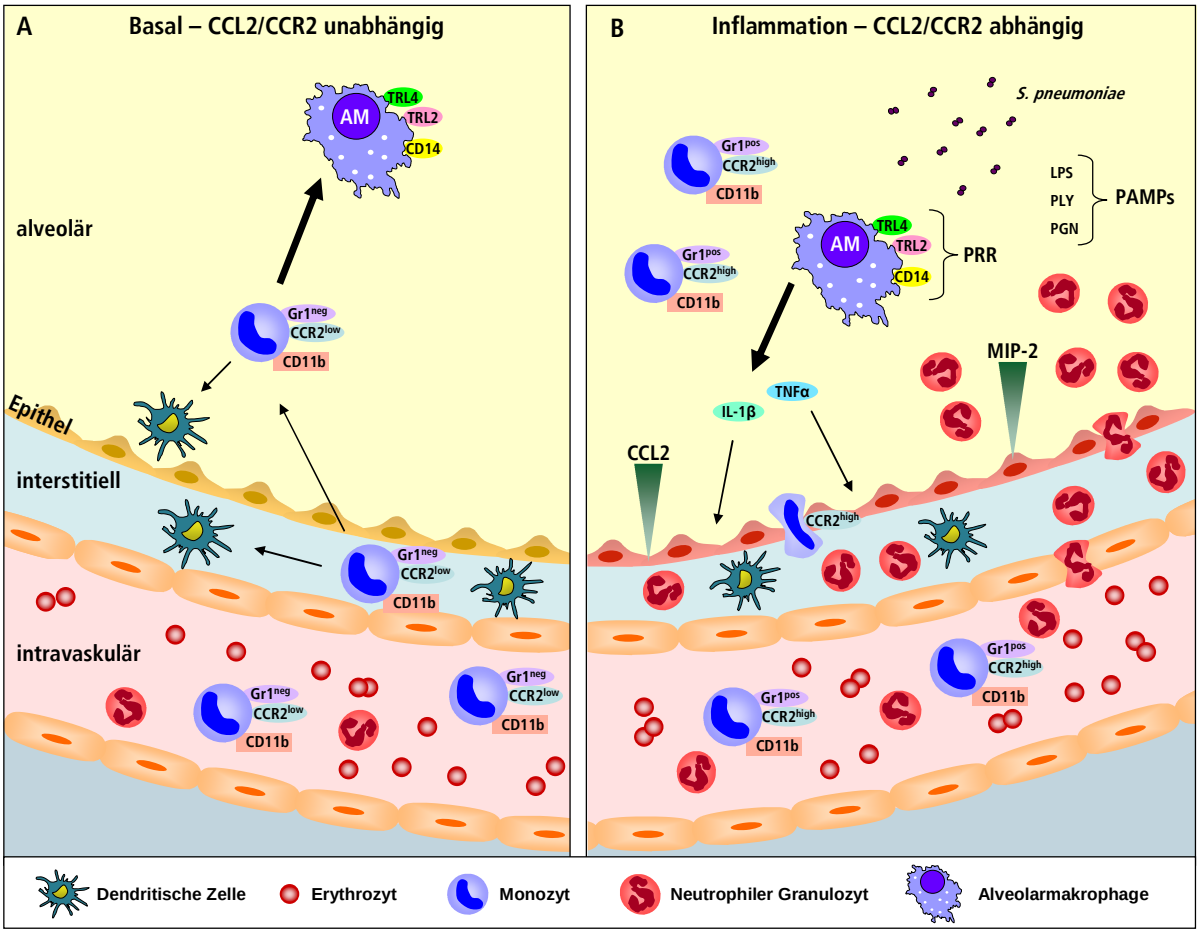


Abb. 2: Monozyten-Subpopulationen des peripheren Blutes und ihre Migration in die Lunge. A) Unter Basalbedingung (keine Inflammation) wandern nicht-inflammatorische Blutmonozyten (Gr-1^{neg}/$CCR2^{neg}$) mit langsamer Kinetik aus dem Kapillarbett in die Lunge, wo sie in Lungenmakrophagen, dendritische Zellen und residente Alveolarmakrophagen differenzieren. B) Unter Entzündungsbedingung werden nach initialer Rekrutierung neutrophiler Granulozyten inflammatorische Monozyten (Gr-1^{pos}/$CCR2^{pos}$) entlang eines CCL2 Chemokingradienten mit rascher Kinetik aus dem Kapillarbett in die Lunge rekrutiert und differenzieren dort in Exsudatmakrophagen (nähere Erläuterungen siehe Text).

1.4 Die CCL2-CCR2 Achse

Die Frühphase der akuten bakteriellen Lungeninfektion ist charakterisiert durch eine früh einsetzende Rekrutierung neutrophiler Granulozyten in das alveoläre Kompartiment und Lungenparenchym, welcher regelhaft eine zeitlich verzögerte Rekrutierung inflammatorischer Monozyten folgt [28]. Für die direktionale Rekrutierung von Leukozyten aus dem Gefäßbett der Lunge in das alveoläre Kompartiment spielen chemotaktisch wirkende Zytokine, sogenannte Chemokine, eine zentrale Rolle [29]. Das CC Chemokin 2 (CCL2, MCP-1, monocyte chemoattractant protein-1) gehört zusammen mit CCL7 und CCL12 (MCP-3, MCP-5) zur Gruppe der CC Chemokine und ist primär für die Rekrutierung inflammatorischer Monozyten verantwortlich, rekrutiert jedoch auch in begrenztem Umfang Lymphozyten-Subsets und natürliche Killerzellen [30-32]. Der Hauptrezeptor von CCL2 ist der CC Chemokin Rezeptor 2 (CCR2), welcher ein G-Protein gekoppelter, seven-transmembrane-spanning (7-TMS) Rezeptor ist (Abb. 3) [33, 34]. Nach der Bindung von CCL2 an CCR2 kommt es im Zuge einer Signaltransduktionskaskade zur downstream von CCR2 lokalisierten Aktivierung der Phosphatidylinositol 3-Kinase γ (PI3Kγ), welche über die Phosphorylierung von Phosphatidylinositol 4,5-diphosphat (PIP2) den „second messenger“ Phosphatidylinositol 3,4,5-triphosphat (PIP3) generiert. PIP3 aktiviert im weiteren Verlauf der Signaltransduktion die downstream agierende Proteinkinase B (PKB). Diese elementaren Signaltransduktionsprozesse nach Bindung von CCL2 an seinen Rezeptor CCR2 führen letztlich zur entzündlich getriebenen Monozyten- bzw. Leukozytenmobilisierung an den Ort des Entzündungsgeschehens [29]. Mit Hilfe spezifischer, oral verfügbarer „small molecule inhibitors“ (AS-605240, AS-604850) zur Blockade von PI3Kγ gelang es kürzlich, im Mausmodell der Kollagen-induzierten Arthritis den Entzündungsprozess in den betroffenen Gelenken massgeblich zu attenuieren [35-37] (Abb. 3).

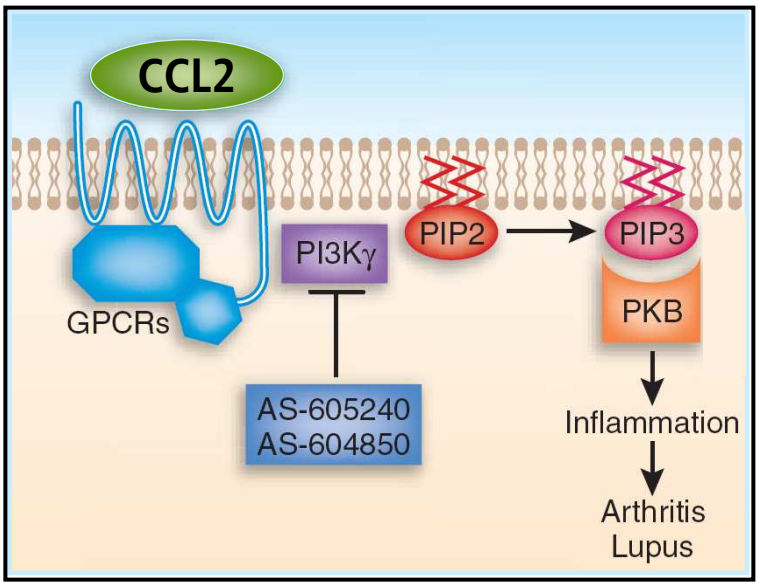


Abb. 3: Schematische Darstellung der CCL2 induzierten Signaltransduktionskaskade. Das Monozyten rekrutierende Chemokin CCL2 bindet an den G-Protein gekoppelten Rezeptor (GPCR) CCR2 und induziert via Aktivierung von PI3Kγ die Bildung von PIP3 mit nachfolgender Aktivierung von PKB als wesentlichen Schritt zur inflammatorisch getriebenen Leukozytenrekrutierung zum Ort der Entzündung (für nähere Erläuterungen siehe Text) (verändert nach Ohashi et al.) [38].

2. Problemstellung der vorliegenden Arbeit

Lungeninfektionen mit *Streptococcus pneumoniae* stellen weltweit aufgrund der hohen Morbidität und Letalität noch immer ein großes medizinisches sowie gesundheitsökonomisches Problem dar. Auf Grund der zunehmenden Entwicklung von Antibiotikaresistenzen gegenüber pneumotropen Krankheitserregern ist es erforderlich, frühzeitig alternative, Antibiotika-unabhängige therapeutische Interventionsmassnahmen zu entwickeln mit dem Ziel, die protektive Immunantwort des Wirtes gegenüber inhalierten bakteriellen Erregern zu verbessern. Eine wesentliche Voraussetzung zum Erreichen eines solchen klinisch relevanten Zieles ist jedoch ein detailliertes Verständnis der den Wirts-Pathogen-Interaktionen zugrunde liegenden molekularen Mechanismen. Gerade die Identifikation mononukleärer Phagozyten-Subsets im peripheren Blut und ihr unterschiedlicher Beitrag zur Homöostase gewebsständiger mononukleärer Phagozyten-Subsets unter nicht-entzündlichen versus akuten Entzündungsbedingungen hat das Verständnis der Pathobiologie mononukleärer Phagozyten im Kontext pulmonaler Infektionen gravierend verkompliziert. Das Ziel der vorliegenden Arbeit ist daher, die Bedeutung des mononukleär-phagozytären Systems in der Pathogenese der Pneumokokken-Pneumonie der Maus zu untersuchen.

Untersuchungen zur Pathobiologie des pneumokokkalen Virulenzfaktors Pneumolysin (PLY) in der akuten Lungenentzündung der Maus haben gezeigt, dass die intratracheale Applikation von PLY in die Lungen gesunder Mäuse eine massive Depletion des residenten Alveolarmakrophagenpools der Lunge induzierte [39]. Da residente Alveolarmakrophagen jedoch die sogenannte ‚first line host defense' der Lunge gegenüber inhalierten Krankheitserregern darstellen, erleichtert ihre Depletion durch Zytolysine wie Pneumolysin die Pneumokokken-Kolonisation des distalen Respirationstraktes und damit die Entwicklung einer Pneumonie [8, 9]. Bakterielle Infektionen der Lunge sind charakterisiert durch eine massive, früh einsetzende Rekrutierung neutrophiler Granulozyten aus dem pulmonalen Kapillarbett in das interstitielle und alveoläre Kompartiment der Lunge. Neutrophile Granulozyten unterstützen residente Alveolarmakrophagen zum einen in der Phagozytose und dem intrazellulären Verdau der Erreger. Zum anderen sind neutrophile Granulozyten zur Produktion reaktiver Sauerstoffspezies befähigt, mit deren Hilfe phagozytierte Erreger intrazellulär abgetötet werden können [29]. Für den Wirt nachteilig wirken

sich jedoch die bakteriziden Eigenschaften neutrophiler Granulozyten dann aus, wenn es aufgrund massiver Neutrophilenrekrutierung und konsekutiver Apoptose/Nekrose-Induktion in dieser kurzlebigen Leukozytenpopulation zur intrapulmonalen Freisetzung reaktiver Sauerstoffspezies kommt, welche beinahe regelhaft eine konsekutive Gewebsschädigung verursachen [40, 41]. Zusammen mit der im Verlauf von Pneumokokkeninfektionen der Lunge zu beobachtenden transienten Alveolarmakrophagen-Depletion wird das Entzündungsgeschehen dahingehend aggraviert, dass apoptotische/nekrotische neutrophile Granulozyten nicht effektiv aus dem alveolären Kompartiment entfernt werden und somit zur Entwicklung ausgedehnter konsolidierter Infiltrate im distalen Respirationstrakt beitragen. Solche konsolidierten Infiltratbildungen im Lungenparenchym *S. pneumoniae*-infizierter Lungen tragen ihrerseits zur Schädigung der Lungenstruktur und demzufolge auch zur Schädigung der Lungenfunktion im Verlauf bakterieller Infektionen bei. Somit könnten therapeutische Interventionen mit dem Ziel einer Verbesserung der Heilungs- und Reparaturphase im Verlauf der Pneumonie entscheidend zur beschleunigten Wiederherstellung der Lungenhomöostase beitragen.

2.1 Fragestellungen

In der vorliegenden Arbeit wurden folgende zentrale Fragestellungen untersucht:

1. Welchen Einfluss hat die ‚downstream' von CCR2 agierende Phosphatidylinositol 3-Kinase γ (PI3Kγ) auf die entzündliche Monozytenrekrutierung sowie die Reparaturphase im Verlauf der Pneumokokken-Pneumonie der Maus?

2. Lässt sich durch konnatale Überexpression des Monozyten rekrutierenden Chemokins CCL2 in der Lunge eine Verstärkung der Wirtsabwehr der Lunge gegenüber einer *Streptococcus pneumoniae* Infektion induzieren?

3. Welchen Effekt hat die pulmonale Expansion dendritischer Zellen auf den Verlauf einer *Streptococcus pneumoniae*-induzierten Pneumonie?

4. Welchen Effekt hat die Deletion von CCL2 auf den Verlauf und Schweregrad einer *Streptococcus pneumoniae* Infektion der Lunge?

3. Ergebnisse

3.1 Einfluss der downstream von CCR2 agierenden Phosphatidylinositol 3-Kinase γ (PI3Kγ) auf die entzündliche Monozytenrekrutierung sowie die Reparaturphase im Verlauf der Pneumokokken-Pneumonie der Maus.

Die Phosphatidylinositol 3-Kinase γ (PI3Kγ) ist eine downstream von G-Protein gekoppelten Rezeptoren (wie z.B. Chemokinrezeptoren) agierende Phospholipidkinase, welche eine zentrale Rolle in der Chemokin-getriebenen entzündlichen Rekrutierung von Leukozyten zum Infektionsort spielt: Während im Modell der *Escherichia coli*-induzierten Peritonitis gezeigt werden konnte, dass die Deletion von PI3Kγ zu einer attenuierten Rekrutierung von Makrophagen in das peritoneale Kompartiment führte [29], liegen bis dato über die Bedeutung der PI3Kγ in der Pathogenese der Pneumokokken-Pneumonie keine Erkenntnisse vor. Daher wurde in der vorliegenden Arbeit untersucht, welchen Effekt die Gendeletion von PI3Kγ oder aber die Applikation oral verfügbarer, PI3Kγ-spezifischer „small-molecule inhibitors“ auf die Wirtsabwehr der Lunge gegenüber *S. pneumoniae* hat.
Histopathologische Untersuchungen der Lungen von *S. pneumoniae* Serotyp 19 (EF3030) infizierten PI3Kγ defizienten (KO) und Wildtyp-Mäusen (WT) zeigten eine stark aggravierte Entzündung in PI3Kγ KO Mäusen, welche charakterisiert war durch die Entwicklung einer eitrigen Pleuritis sowie einer ausgeprägten konsolidierten Infiltratbildung mit ausgedehnter intra-alveolärer Deposition nekrotischer neutrophiler Granulozyten (Abb. 3 in der Anlage 1). Analysen zur Erregerelimination in der bronchoalveolären Lavageflüssigkeit (BAL) von PI3Kγ KO und WT Mäusen bzw. Mäusen, die mit dem spezifischen „small-molecule inhibitor“ AS-605240 gegen PI3Kγ vorbehandelt wurden, ergaben, dass PI3Kγ KO Mäuse sowie AS-605240 vorbehandelte WT Mäuse im Vergleich zu WT Mäusen eine signifikant reduzierte Erregerelimination zeigten (Abb. 4A und 4B; Abb. 4 in der Anlage 1). Diese verminderte Erregerelimination in PI3Kγ KO Mäusen und AS-605240 vorbehandelten WT Mäusen war jedoch nicht auf eine reduzierte Rekrutierung neutrophiler Granulozyten zurückzuführen: die Zelldifferenzierung in Pappenheim-gefärbten Zytozentrifugenpräparaten von Pneumokokken-infizierten PI3Kγ KO und AS-605240 vorbehandelten WT Mäusen zeigte nämlich keine signifikanten Unterschiede in der

Zellzahl rekrutierter neutrophiler Granulozyten im Vergleich zu infizierten Wildtyp-Mäusen (Abb. 4 in der Anlage 1).

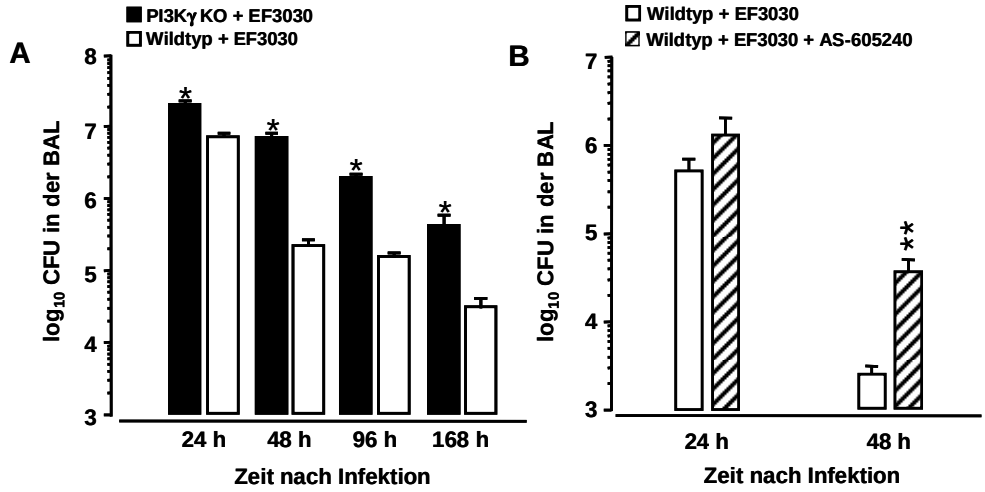


Abb. 4: Analyse der Erregerelimination in *S. pneumoniae*-infizierten PI3Kγ KO und Wildtyp-Mäusen bzw. AS-605240 vorbehandelten Wildtyp-Mäusen. (A) Keimlast (CFU) in der BAL *S. pneumoniae*-infizierter PI3Kγ KO Mäuse und WT Mäuse. (B) Keimlast (CFU) in der BAL *S. pneumoniae*-infizierter WT und AS-605240 vorbehandelter WT Mäuse.

Durchflusszytometrische Analysen der BAL-Zellen unbehandelter und *S. pneumoniae*-infizierter PI3Kγ KO und WT Mäuse bzw. AS-605240 vorbehandelter WT Mäuse ergaben unter Basalbedingungen vergleichbare Zellzahlen residenter Alveolarmakrophagen. Jedoch zeigten insbesondere PI3Kγ KO Mäuse im Vergleich zu Wildtyp-Mäusen eine starke Depletion des Alveolarmakrophagenpools innerhalb der ersten 24 h nach *S. pneumoniae* Infektion. Bereits 48 h nach Infektion mit *S. pneumoniae* war der Alveolarmakrophagenpool in WT Mäusen re-expandiert, während im Vergleich hierzu PI3Kγ KO Mäuse signifikant geringere Alveolarmakrophagenzahlen in der BAL zeigten (Abb. 4C in der Anlage 1). Darüber hinaus ließ sich nachweisen, dass die aus inflammatorischen Monozyten hervorgehenden, neu rekrutierten Exsudatmakrophagen (F/480^{pos}, CD11c^{pos} und CD11b^{pos}) (Abb. 5A; Abb. 5 in der Anlage 1) im Vergleich zu korrespondierend infizierten WT Mäusen in signifikant geringeren Zellzahlen in die Lungen Pneumokokken-infizierter PI3Kγ KO Mäuse sowie auch AS-605240 vorbehandelter WT Mäuse rekrutiert wurden (Abb. 5B und 5D; Abb. 5 in der Anlage 1).

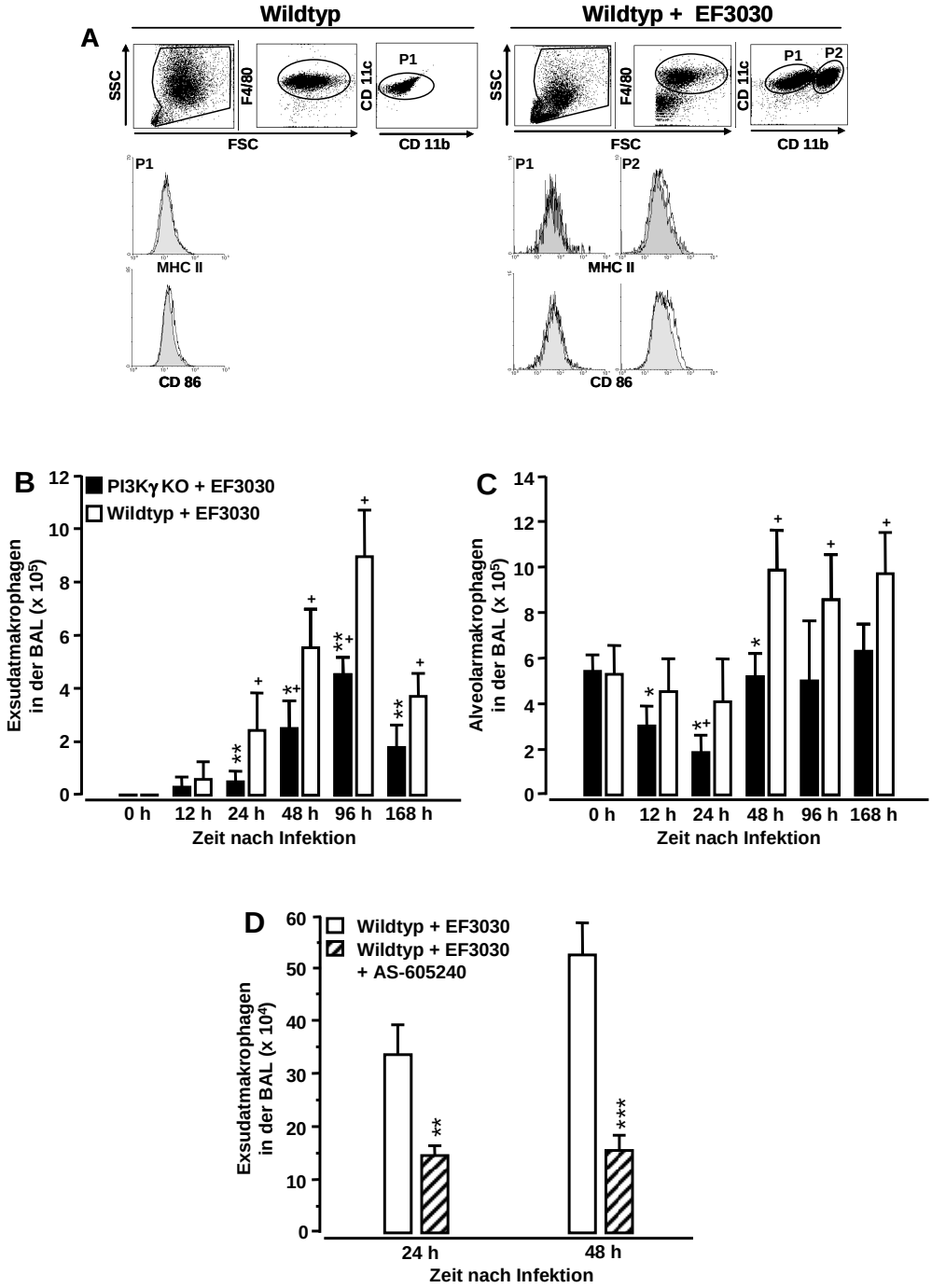


Abb. 5: Effekt der Deletion oder Inhibition von PI3Kγ auf die alveoläre Rekrutierung mononukleärer Phagozyten in der Pneumokokken-Pneumonie der Maus. (A) Durchflusszytometrische Analyse von Alveolarmakrophagen (P1) und Exsudatmakrophagen (P2) in der BAL von schein-infizierten (mock-infected) Wildtyp-Mäusen und *S. pneumoniae*-infizierten Wildtyp-Mäusen. (B, C) Auf FACS-Analysen basierende Quantifizierung alveolär rekrutierter Exsudatmakrophagen (B) und Alveolarmakrophagen (C) in der bronchoalveolären Lavageflüssigkeit von PI3Kγ KO und WT Mäusen. (D) Quantifizierung der Exsudatmakrophagenrekrutierung in Wildtyp-Mäusen in An- und Abwesenheit des PI3Kγ-spezifischen Inhibitors AS-605240.

Schließlich wurde in diesem Teil der Arbeit auch der Effekt der Deletion von PI3Kγ auf das Überleben von Mäusen nach Infektion mit dem invasiv wachsenden *S. pneumoniae* Serotyp 2 (D39) analysiert. Interessanterweise zeigte sich, dass PI3Kγ KO Mäuse ein signifikant reduziertes Überleben im Vergleich zu D39 infizierten WT Mäusen aufwiesen (Abb. 6; Abb. 7 in der Anlage 1).

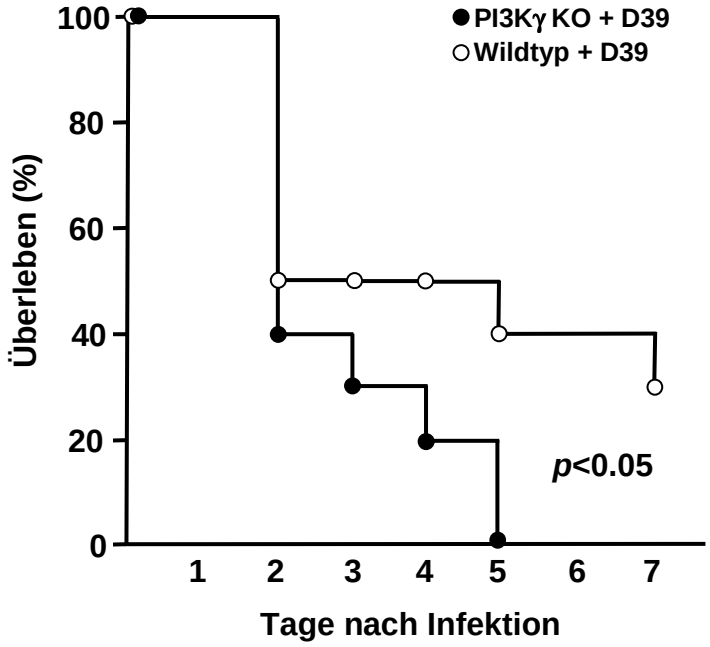


Abb. 6: Analyse des Überlebens von PI3Kγ KO und WT Mäusen nach Infektion mit *S. pneumoniae* D39. PI3Kγ KO und WT Mäuse (n=10 pro Gruppe) wurden intratracheal mit *S. pneumoniae* D39 infiziert und das Überleben über einen Zeitraum von 7 Tagen analysiert.

3.2 Verstärkung der Wirtsabwehr der Lunge gegenüber einer *S. pneumoniae* Infektion durch konnatale Überexpression des Monozyten rekrutierenden Chemokins CCL2 in der Lunge

In diesem Teil der Arbeit wurden CCL2 transgene (CCL2 tg) Mäuse verwendet, in denen das Monozyten rekrutierende Chemokin CCL2 in Typ II Alveolarepithelzellen unter der Kontrolle des Surfactant Protein C (SP-C) Promoters überexprimiert ist und kontinuierlich in das alveoläre Kompartiment sezerniert wird. Als Konsequenz dieser konstitutiven lungenspezifischen Überexpression von CCL2 zeigen CCL2 tg Mäuse eine verstärkte Rekrutierung mononukleärer Phagozyten-Subsets in das alveoläre Kompartiment und Lungenparenchym [42]. Aufgrund der im zuvor beschriebenen

PI3Kγ-Modell gewonnen Erkenntnisse formulierten wir die Hypothese, dass ein bereits vor der Infektion expandierter mononukleärer Phagozytenbesatz der Lunge die pulmonale Infektabwehr gegenüber *S. pneumoniae* verbessert. In ersten Experimenten wurde untersucht welchen Effekt die CCL2 Überexpression auf die Zusammensetzung mononukleärer Phagozyten-Subsets im bronchoalveolären Kompartiment hat. In Pappenheim-gefärbten Zytozentrifugenpräparaten von Zellen der bronchoalveolären Lavageflüssigkeit (BAL) von Wildtyp und CCL2 tg Mäusen konnten residente Alveolarmakrophagen als zellulärer Hauptbestandteil der BAL identifiziert werden. Darüber hinaus ließen sich in der BAL CCL2 tg Mäuse Zellen mit monozytärer Morphologie identifizieren (Abb. 7A; Abb. 1 in der Anlage 2). Zur detaillierten Charakterisierung der BAL-Zellen wurden durchflusszytometrische Analysen der zellulären Oberflächenantigenexpressionsmuster der individuellen Phagozyten-Subsets und eine auf diesen Analysen beruhende Quantifizierung dieser Zellpopulationen durchgeführt (Abb. 7B-7E; Abb. 1 in der Anlage 2). Nach diesen durchflusszytometrischen Analysen zeigten unbehandelte CCL2 tg Mäuse eine F/480^{pos}, CD11c^{pos}, CD11b^{neg} Population (P1, Abb. 7B, Abb. 1 in der Anlage 2), welche MHCII^{neg}, CD86^{neg} und CD49d^{neg} war und somit dem klassischen Immunphänotyp residenter Alveolarmakrophagen entspricht (Abb. 7C; Abb. 1 in der Anlage 2) [27, 43]. Neben residenten Alveolarmakrophagen (P2, Abb. 7B; Abb. 1 in der Anlage 2) zeigten CCL2 tg Mäuse außerdem eine Population myeloider dendritischer Zellen (DC) mit dem Expressionsprofil F/480^{pos}, CD11c^{high}, CD11b^{high}, MHCII^{high}, CD86^{pos}, CD49d^{pos}, Gr-1^{neg} (P3, Abb. 7B und 7C; Abb. 1 in der Anlage 2) sowie eine Population alveolärer Monozyten mit dem Expressionsprofil F/480^{pos}, CD11c$^{neg\text{-}mid}$, CD11b^{high}, MHCII$^{neg\text{-}low}$, CD86^{low}, CD49d^{pos}, Gr-1$^{low\text{-}mid}$ (P4, Abb. 7B und 7C; Abb. 1 in der Anlage 2). Zusammengefasst zeigen diese durchflusszytometrischen Analysen, dass CCL2 tg Mäuse im Vergleich zu Wildtyp-Mäusen bereits unter Basalbedingungen gesteigerte Zellzahlen residenter Alveolarmakrophagen, dendritischer Zellen und alveolär rekrutierter Monozyten aufweisen (Abb. 7D und 7E; Abb. 1 in der Anlage 2).

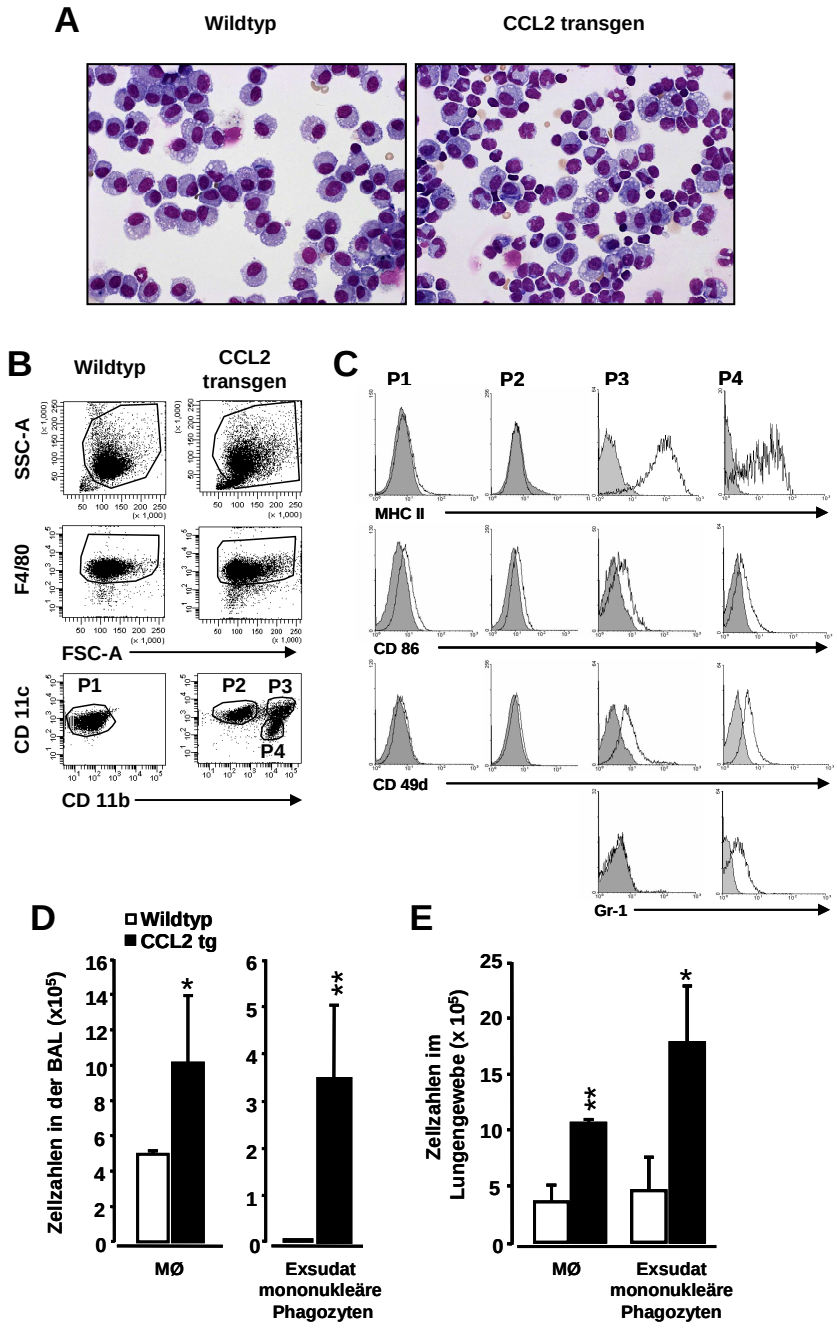


Abb. 7: Effekt der lungenspezifischen CCL2 Überexpression auf die Akkumulation mononukleärer Phagozyten in der Maus. (A) Pappenheim-gefärbte Zytozentrifugenpräparate der BAL-Zellen von Wildtyp-Mäusen und CCL2 transgenen Mäusen. (B, C) Durchflusszytometrische Analyse der BAL-Zellen von unbehandelten Wildtyp-Mäusen und CCL2 tg Mäusen. Die Zellen wurden nach ihren FSC/SSC- und FSC/F4/80 Charakteristika „gegated" und anschliessend einer Oberflächenantigenexpressionsanalyse unterworfen. (D) Quantifizierung der Alveolarmakrophagen (MØ) und Exsudat-mononukleären Phagozyten in der BAL und (E) im Lungenparenchym. Für weitere Details siehe Text bzw. Anlage 2.

Die Analyse der bakteriellen Clearance in *S. pneumoniae* Serotyp 19 (EF3030) infizierten CCL2 tg und WT Mäusen ergab eine signifikant bessere Erregerelimination in CCL2 transgenen Mäusen im Vergleich zu WT Mäusen (Abb. 8A; Abb.2 in der Anlage 2). Parallel zum Auswachsen der Keime in den Lungen der Wildtyp-Mäuse zeigte sich beginnend ab Tag 2 nach Infektion eine signifikant höhere Letalität in *S. pneumoniae*-infizierten Wildtyp-Mäusen im Vergleich zu CCL2 tg Mäusen. An Tag 7 nach Infektion zeigten CCL2 tg Mäuse lediglich eine Letalität von ~20%, während Wildtyp-Mäuse eine Letalität von ~65% aufwiesen (Abb. 8B; Abb. 2 in der Anlage 2). Histopathologische Untersuchungen der Lungen *S. pneumoniae*-infizierter CCL2 tg Mäuse versus WT Mäuse zeigten eine deutlich aggravierte Lungenentzündung in Wildtyp-Mäusen bereits an Tag 3 nach Infektion (Abb.8C; Abb. 2 in der Anlage 2), was auf eine schlechtere Heilungs- und Reparaturphase in dieser experimentellen Gruppe im Vergleich zu CCL2 tg Mäusen hindeutet.

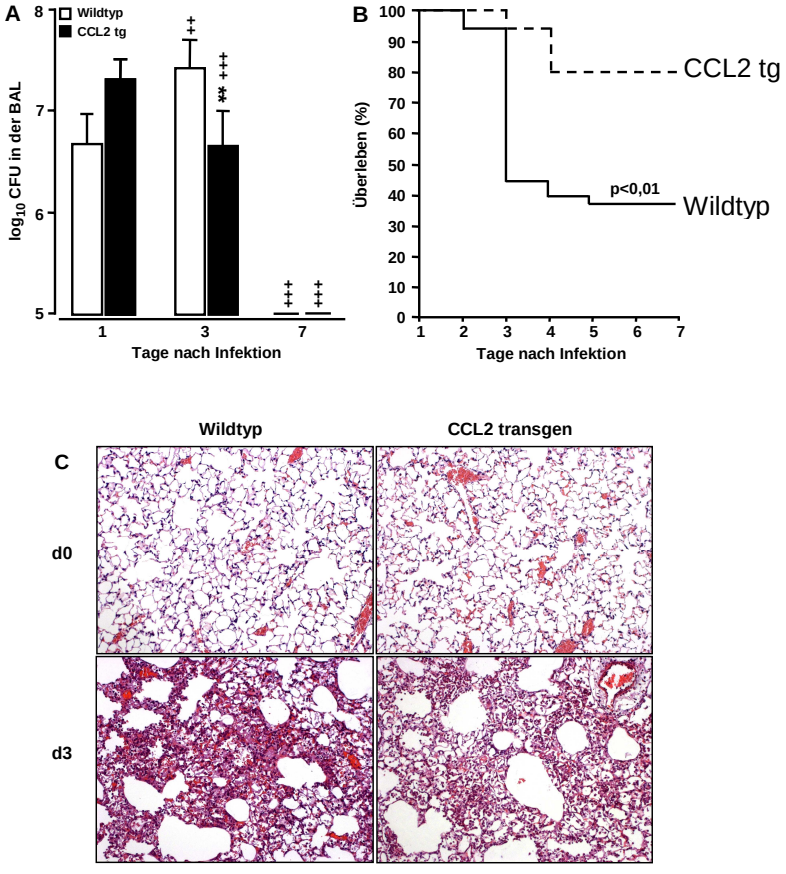


Abb. 8: Erregerelimination und Überleben in *S. pneumoniae*-infizierten Wildtyp-Mäusen und CCL2 tg Mäusen. (A) Keimlast in der BAL Pneumokokken-infizierter Wildtyp-Mäuse und CCL2 tg Mäuse. (B) Überleben (in %) von Wildtyp-Mäusen und CCL2 tg Mäusen nach Infektion mit *S. pneumoniae*.

Auf Grund dieser deutlichen Unterschiede in der Infektabwehr zwischen WT Mäusen und CCL2 tg Mäusen wurde eine durchflusszytometrische Analyse der unter *S. pneumoniae* Infektion in die Lunge rekrutierten Phagozytensubsets durchgeführt. Bereits 24 h nach Infektion zeigten sowohl Wildtyp-Mäuse als auch CCL2 tg Mäuse eine transiente Depletion des residenten Alveolarmakrophagenpools, welche jedoch in CCL2 tg Mäusen im Vergleich zu Wildtyp-Mäusen weniger stark ausgeprägt war. Ab Tag 7 nach Infektion liess sich eine Re-Expansion des Alveolarmakrophagenpools nachweisen (Abb. 9A; Abb. 5 in der Anlage 2), wobei insbesondere CCL2 tg Mäuse während des gesamten Untersuchungszeitraumes eine verstärkte Mobilisierung mononukleärer Phagozyten im Vergleich zu Wildtyp-Mäusen zeigten, welches die bereits vorab beschriebene, verbessert ablaufende Reparaturphase in den CCL2 transgenen Mäusen unterstützt (Abb. 9B; Abb. 5 in der Anlage 2).

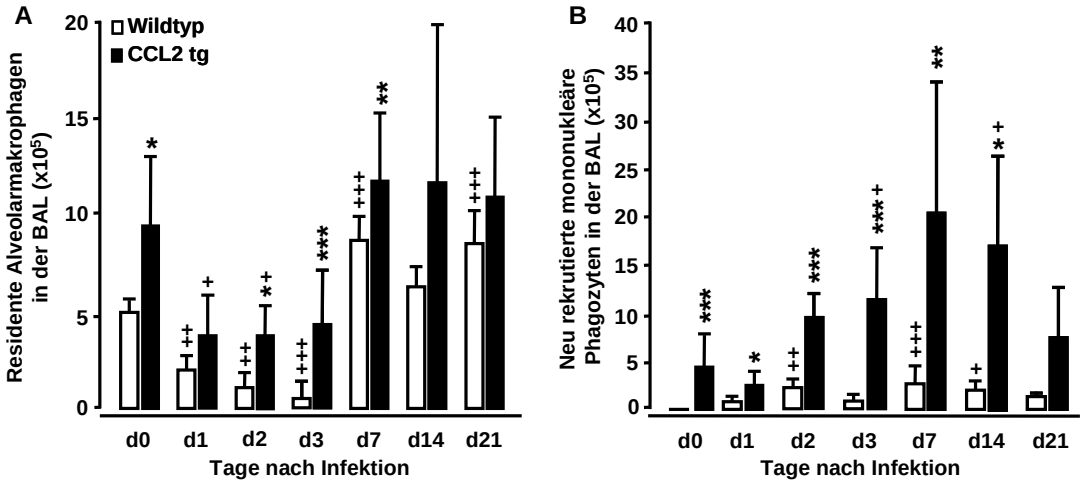


Abb. 9: Rekrutierung mononukleärer Phagozyten in *S. pneumoniae*-infizierten CCL2 tg Mäusen und Wildtyp-Mäusen. (A) Quantifizierung residenter Alveolarmakrophagen und neu rekrutierter mononukleärer Phagozyten (B) in der BAL *S. pneumoniae*-infizierter Wildtyp-Mäuse und CCL2 tg Mäuse.

Eine wesentliche klinische Komplikation in der Spätphase pulmonaler Infektionen sowie auch des pneumogenen ARDS sind fibroproliferative Umbauprozesse des Lungenparenchyms, welche sowohl die Struktur als auch die Gasaustauschfunktion der Lunge nachhaltig beeinträchtigen können [44]. Interessanterweise zeigte die histopathologische Untersuchung des Lungengewebes *S. pneumoniae*-infizierter CCL2 tg Mäuse an Tag 7 nach Infektion die Entwicklung einer Bronchiolitis obliterans (Abb. 10A; Abb. 6 in der Anlage 2). Die Quantifizierung dieser fibrotischen Läsionen

ergab eine signifikant höhere Anzahl obliterierter terminaler Bronchiolen in CCL2 tg Mäusen im Vergleich zu Wildtyp-Mäusen (Abb. 10B; Abb. 6 in der Anlage 2).

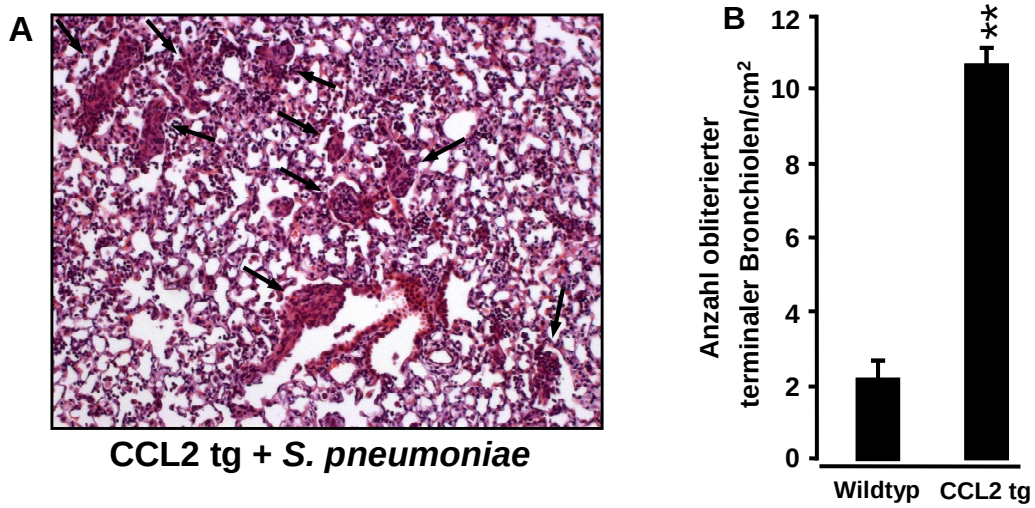


Abb. 10: Nachweis fibroproliferativer Umbauprozesse (Bronchiolitis obliterans) in den Lungen *S. pneumoniae*-infizierter CCL2 transgener Mäuse. (A) Histopathologie von Lungenschnitten CCL2 überexprimierender Mäuse an Tag 7 nach Infektion mit *S. pneumoniae*. Gelbe Pfeile zeigen obliterierte terminale Bronchiolen. (B) Quantifizierung der Bronchiolitis obliterans in *S. pneumoniae*-infizierten Wildtyp-Mäusen und CCL2 tg Mäusen an Tag 7 nach Infektion.

Um zu analysieren, inwieweit die Entwicklung einer Bronchiolitis obliterans in den Lungen *S. pneumoniae*-infizierter CCL2 tg Mäuse auf CCL2-CCR2 abhängige zelluläre Rekrutierungsprozesse zurückzuführen ist, wurden Wildtyp und CCL2 tg Mäuse vor und während der *S. pneumoniae* Infektion mit einem funktionsblockierenden Antikörper gegen CCR2 (MC21) vorbehandelt [26, 39]. Die hierdurch induzierte Blockade der Interaktion des Chemokins CCL2 mit seinem Rezeptor CCR2 führte zwar zu einer kompletten Inhibition der Bronchiolitis obliterans Entwicklung in CCL2 tg Mäusen, jedoch zur gleichen Zeit ebenso zu einer signifikant reduzierten inflammatorischen Exsudatmakrophagenrekrutierung koinzident mit einer massiv beeinträchtigten Reparaturphase an Tag 7 nach Infektion (Abb. 6 in der Anlage 2).

3.3 Effekt der pulmonalen Expansion dendritischer Zellen auf den Verlauf einer *Streptococcus pneumoniae* induzierten Pneumonie

Der vorab beschriebene Befund einer verbesserten Wirtsabwehr der Lunge gegenüber *S. pneumoniae* in CCL2 überexprimierenden Mäusen liess die Frage offen, welchen Beitrag individuelle mononukleäre Phagozyten-Subsets in der Pathogenese der Pneumokokken-Pneumonie spielen. Vor diesem Hintergrund wurden Untersuchungen zur Pathobiologie dendritischer Zellen in der *S. pneumoniae*-induzierten Lungenentzündung durchgeführt. Bislang ist die Funktion dendritischer Zellen in der Pathogenese der Pneumokokken-Pneumonie völlig unbekannt. Dendritische Zellen sind strategisch zwischen Alveolarepithel und Kapillarendothel lokalisiert und sind somit möglicherweise aufgrund ihrer mikroanatomischen Lokalisation prädestiniert, die Wirtsabwehr der Lunge gegenüber inhalierten bakteriellen Erregern zu unterstützen. Daher wurden in der vorliegenden Arbeit Mäuse mit dem DC-spezifischen Wachstumsfaktor Fms-like tyrosine kinase-3 ligand (Flt3L) vorbehandelt. Hierbei führt die wiederholte subkutane Applikation von Flt3L in Mäusen im Lungeninterstitium und in anderen Organsystemen zu einer verstärkten Akkumulation dendritischer Zellen [45]. In initialen Experimenten wurde zunächst analysiert, welchen Effekt die Flt3L Vorbehandlung auf Monozyten-Subsets in der Lunge hat. Interessanterweise konnte gezeigt werden, dass Flt3L vorbehandelte Mäuse eine transiente Monozytose im peripheren Blut entwickelten. Demgegenüber ergab die auf durchflusszytometrischen Analysen basierende Quantifizierung der diversen pulmonalen Phagozyten-Subsets keine Unterschiede hinsichtlich Alveolarmakrophagen, alveolären Exsudatmakrophagen bzw. Lungenmakrophagen (Abb. 11A-11C; Abb. 1 in der Anlage 3), wohingegen die Anzahl pulmonaler myeloider DC in Flt3L vorbehandelten Mäusen signifikant höher war als in Kontrollmäusen (Abb. 11D; Abb. 1 in der Anlage 3).

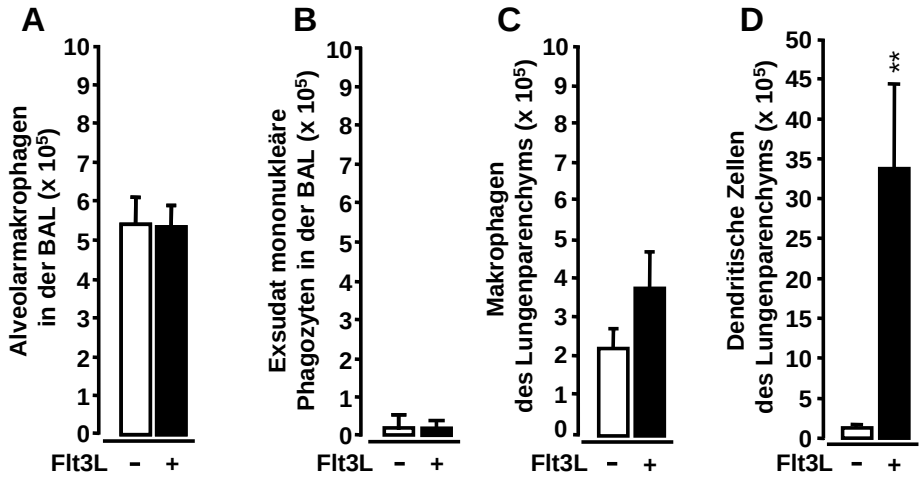


Abb. 11: Der Wachstumsfaktor Flt3L induziert die Akkumulation myeloider dendritischer Zellen (DC) im Lungenparenchym von Mäusen. Auf FACS-Analysen basierende Quantifizierung alveolärer Makrophagen (A), Exsudat-mononukleärer Phagozyten in der BAL (B), Lungenmakrophagen (C) sowie dendritischer Zellen des Lungenparenchyms (D). Für Details siehe Anlage 3.

Weiterhin zeigte sich, dass die Infektion Flt3L vorbehandelter Mäuse mit *S. pneumoniae* im Vergleich zu *S. pneumoniae*-infizierten Kontrollmäusen zu einer massiv aggraviert verlaufenden Lungenentzündung führte, welche charakterisiert war durch die Entwicklung einer Vaskulitis und Mikrothrombenbildung im Gefäßbett sowie einer ausgeprägten Zunahme der Lungenpermeabilität bereits an Tag 2 nach Infektion mit einem Maximum an Tag 3 (Abb. 12A-12D; Abb. 2 in der Anlage 3).

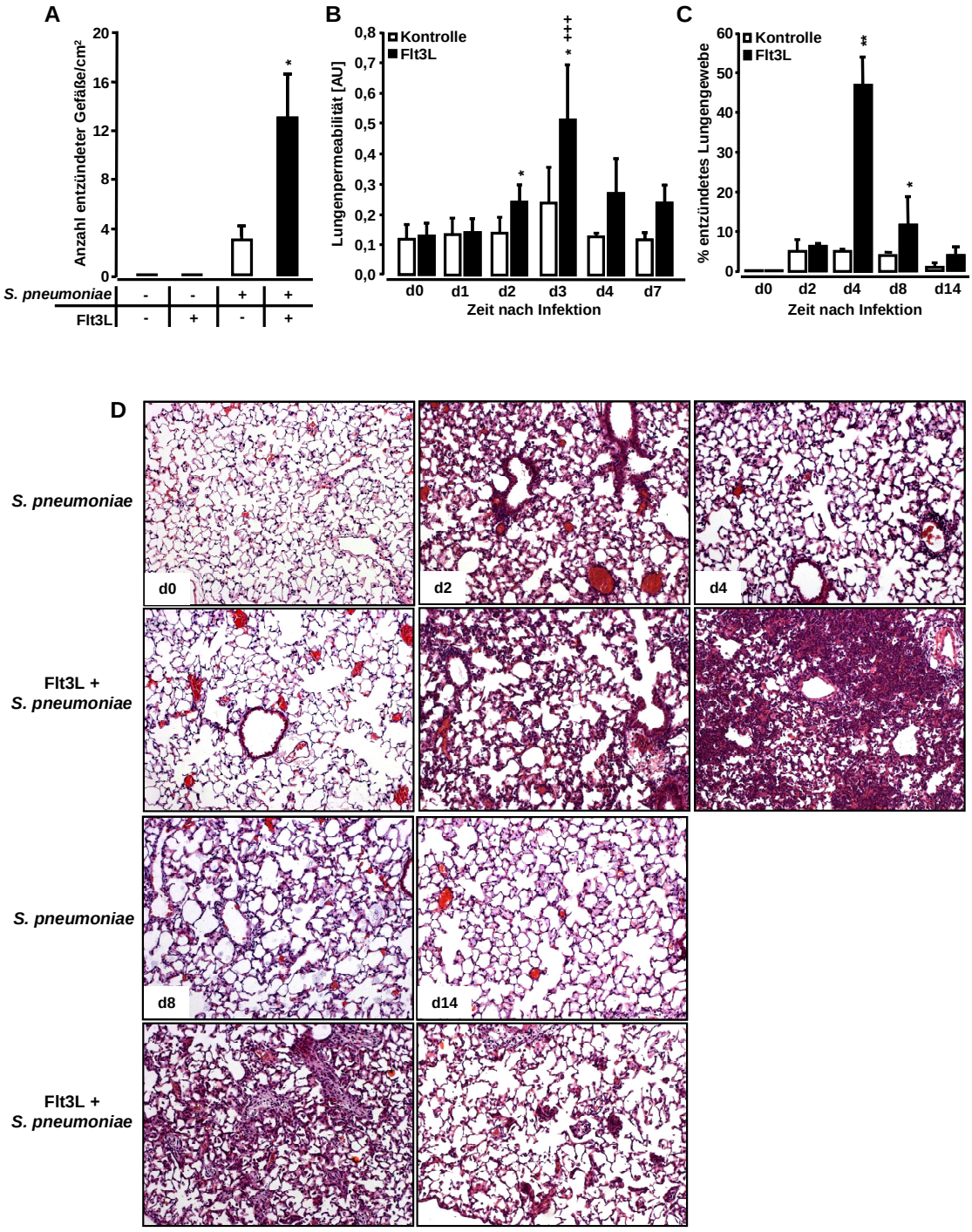


Abb. 12: Vaskulitis und Lungenpermeabilität in Flt3L vorbehandelten, *S. pneumoniae*-infizierten Mäusen. (A) Quantifizierung der Vaskulitis in Histologien von Lungen Flt3L vorbehandelter, *S. pneumoniae*-infizierter Mäuse und Kontrollmäuse an Tag 2 nach Infektion. (B) Messungen der Lungenpermeabilität in Flt3L vorbehandelten, *S. pneumoniae*-infizierten Mäusen und Kontrollmäusen. (C) Quantifizierung des Lungengewebsschadens in (D) Histopathologien von Flt3L vorbehandelten, *S. pneumoniae*-infizierten Mäusen und Kontrollmäusen.

Die Analyse der Erregerelimination im Lungenparenchym Flt3L vorbehandelter Mäuse ergab eine anhaltend hohe Erregerlast bis zu Tag 4 nach Infektion, wohingegen zu diesem Zeitpunkt in Kontrollmäusen keine Erreger mehr nachgewiesen werden konnten (Abb. 13A und 13B; Abb. 3 in der Anlage 3). Diese deutlichen Unterschiede in der Infektabwehr von *S. pneumoniae* resultierten schließlich in einer signifikant höheren Letalität der Flt3L vorbehandelten Mäuse im Vergleich zu Kontoll-vorbehandelten, *S. pneumoniae*-infizierten Wildtyp-Mäusen (Abb. 13C; Abb. 3 in der Anlage 3).

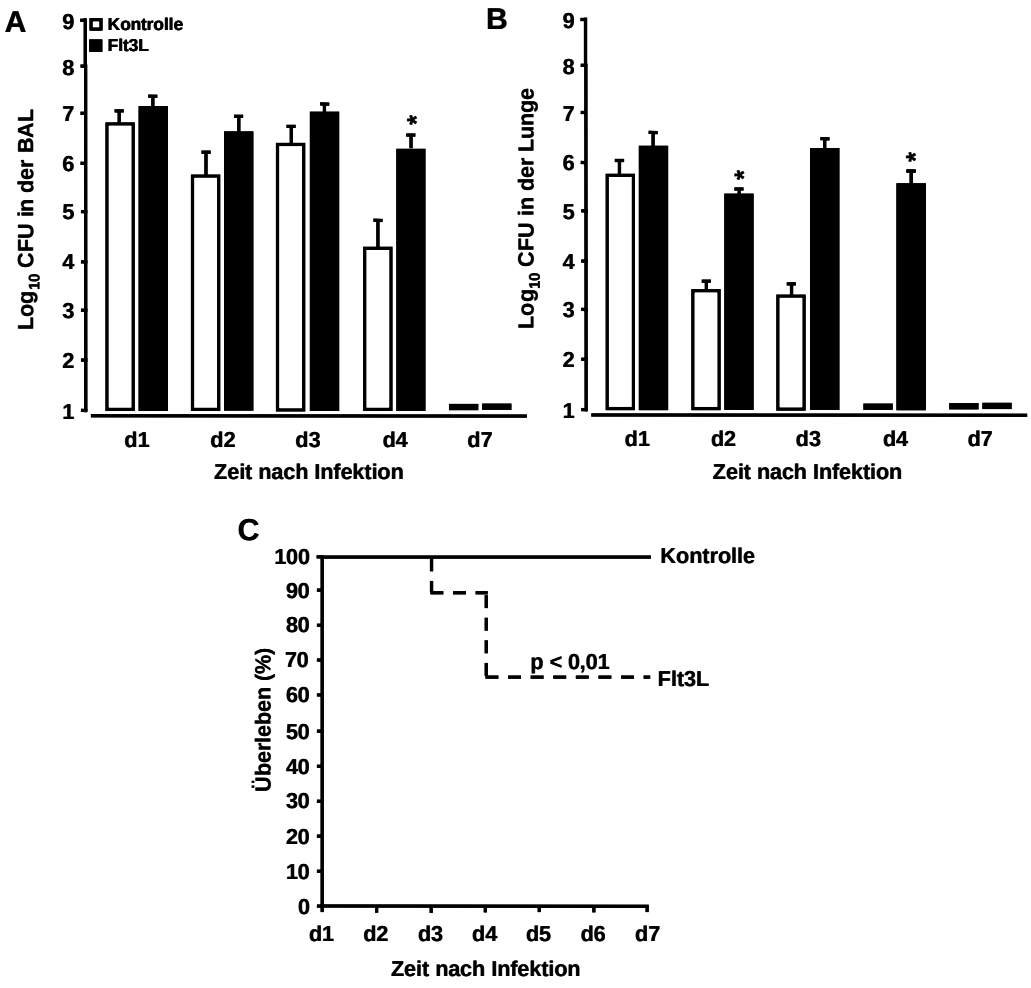


Abb. 13: Erregerlast und Überleben in Flt3L vorbehandelten, *S. pneumoniae*-infizierten Mäusen und Kontrollmäusen. (A) Keimzahlen (CFU) in der bronchoalveolären Lavageflüssigkeit und (B) im Lungenparenchym in Flt3L vorbehandlten, *S. pneumoniae*-infizierten Mäusen und Kontrollmäusen. (C) Überleben von Flt3L vorbehandlten, *S. pneumoniae*-infizierten Mäusen und Kontrollmäusen.

Die Analyse der Alveolarmakrophagenzahlen in der bronchoalveolären Lavageflüssigkeit Flt3L vorbehandelter Mäuse sowie Kontrollmäuse ergab 2 und 3 Tage nach Infektion zunächst eine Depletion des Alveolarmakrophagenpools, welcher eine Re-Expansion der Alveolarmakrophagen zu Tag 7 post Infektion folgte (Abb. 14A; Abb. 5 in der Anlage 3), während die Zahl der Lungenmakrophagen in beiden experimentellen Gruppen kontinuierlich zunahm (Abb. 14B; Abb. 5 in der Anlage 3). Außerdem zeigten Flt3L vorbehandelte Mäuse eine starke Mobilisierung neu rekrutierter Exsudatmakrophagen ($CD11c^{pos}$, $CD11b^{pos}$, $MHCII^{neg}$ und $CD86^{neg}$) im Vergleich zu *S. pneumoniae*-infizierten Kontrollmäusen (Abb. 14C; Abb. 5 in der Anlage 3). FACS-Analysen pulmonaler dendritischer Zellen in den Lungen Flt3L vorbehandelter, *S. pneumoniae* infizierter Mäuse zeigten, ausgehend von einer starken basalen DC Akkumulation im Lungenparenchym bereits einen Tag nach Infektion mit *S. pneumoniae* eine starke Abnahme der DC-Zahlen, gefolgt von einem starken Anstieg pulmonaler DCs an Tag 4 und Tag 7 nach Infektion. Demgegenüber mobilisierten *S. pneumoniae*-infizierte Kontrollmäuse über den gesamten Infektionsverlauf deutlich weniger myeloide DCs (Abb. 14D; Abb. 5 in der Anlage 3). Die Analyse der Pneumokokken-induzierten Mobilisierung neutrophiler Granulozyten in das alveoläre Kompartiment Flt3L-vorbehandelter Mäuse zeigte bis Tag 3 nach Infektion keine Unterschiede zwischen den experimentellen Gruppen, wohingegen die Anzahl neutrophiler Granulozyten in der BAL von Kontrollmäusen zu Tag 4 und 7 abnahm, während Flt3L-vorbehandelte Mäuse eine bis Tag 7 anhaltend gesteigerte Neutrophilenrekrutierung in den Alveolarraum der Lunge aufwiesen (Abb. 14E; Abb. 5 in der Anlage 3).

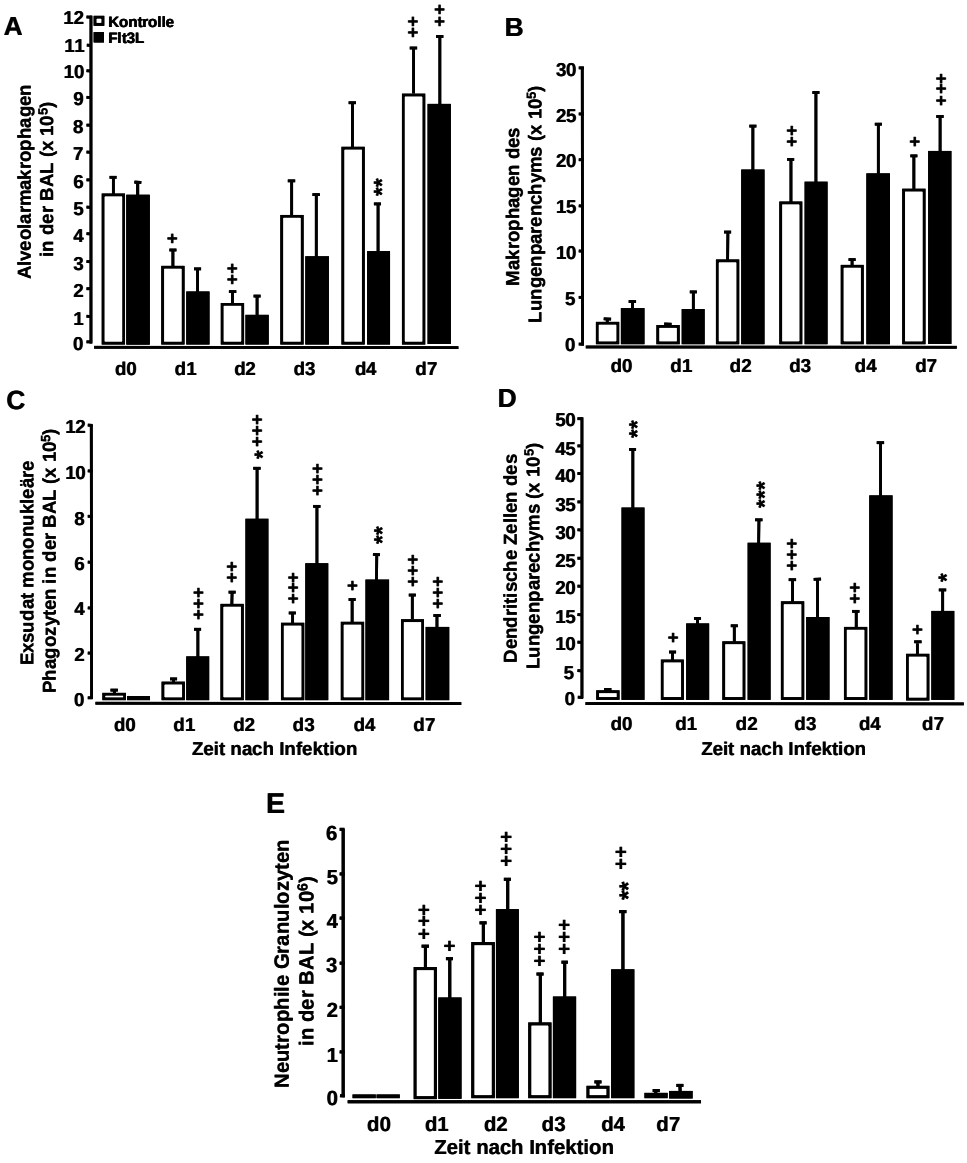


Abb. 14: Pulmonale Leukozytenrekrutierung in Flt3L vorbehandelten Mäusen versus Kontrollmäusen nach Infektion mit *S. pneumoniae*. Quantifizierung der Alveolarmakrophagen (A), Lungenmakrophagen (B), alveolären Exsudatmakrophagen (C), Lungen-DCs (D) und neutrophilen Granulozyten in der BAL (E).

Wie vorab beschrieben entwickelten Flt3L vorbehandelte Mäuse als Antwort auf Infektion mit *S. pneumoniae* eine Vaskulitis sowie gesteigerte Lungenpermeabilität und konsekutiv erhöhte pulmonale Rekrutierung mononukleärer Phagozyten im Vergleich zu Kochsalz-vorbehandelten Kontrollmäusen. Um die Frage zu klären, ob die periphere Monozytose und konsekutiv induzierte verstärkte Mobilisierung mononukleärer Phagozyten möglicherweise für die Vaskulitis und Lungenpermeabilitätserhöhung in *S. pneumoniae*-infizierten Flt3L vorbehandelten

Mäusen verantwortlich ist, wurden Flt3L vorbehandelte Mäuse und Kontrollmäuse vor und während der Infektion mit *S. pneumoniae* mit einem funktionsblockierenden Antikörper mit Spezifität gegen den Chemokinrezeptor CCR2 (MC21) vorbehandelt. Messungen der Lungenpermeabilität in MC21 behandelten Mäusen zeigten, dass die Applikation von MC21 die Lungenpermeabilitätserhöhung in Flt3L vorbehandelten Mäusen signifikant verringerte im Vergleich zu Flt3L vorbehandelten Mäusen nach Applikation von Kontrollantikörper (Abb. 15A; Abb. 7 in der Anlage 3). Gleichzeitig attenuierte die Applikation von MC21 die Rekrutierung von Exsudatmakrophagen, Lungenmakrophagen und DCs sowohl in Flt3L vorbehandelten als auch Kontrollmäusen nach Infektion mit *S. pneumoniae* (Abb. 15B-15D; Abb. 7 in der Anlage 3).

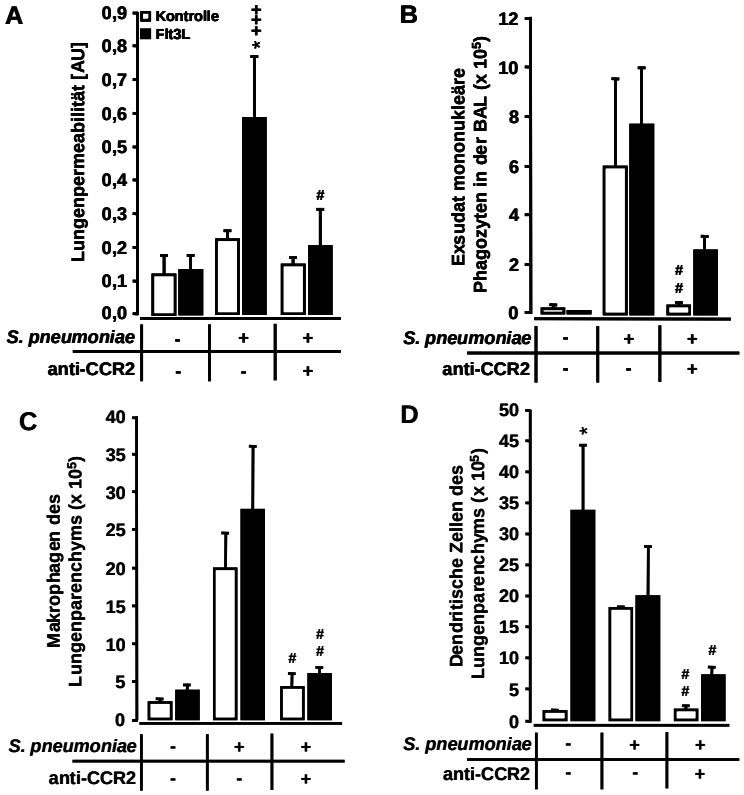
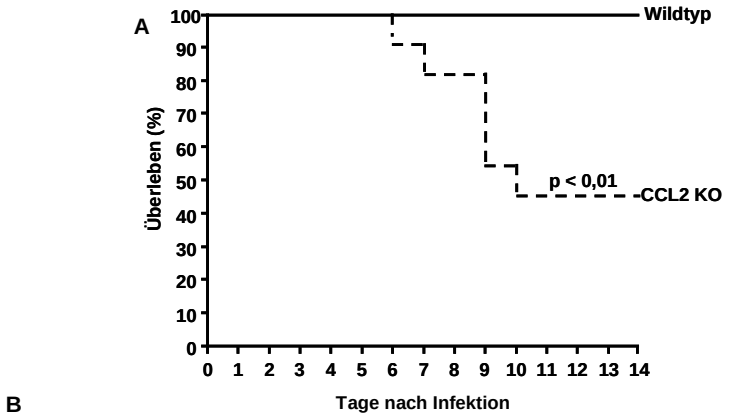


Abb. 15: Effekt der CCR2 Blockade auf die Lungenpermeabilität und entzündliche Rekrutierung mononukleärer Phagozyten in die Lungen Flt3L vorbehandelter Mäuse sowie Kontrollmäuse. (A) Lungenpermeablität in Kontrollmäusen und Flt3L vorbehandelten Mäusen nach Infektion mit *S. pneumoniae* in An- und Abwesenheit des CCR2-Antikörpers MC21 (Tag 3). (B) Alveoläre Rekrutierung von Exsudatmakrophagen (Tag 3) nach Infektion mit *S. pneumoniae* in An- und Abwesenheit von MC21. (C) Rekrutierung von Lungenmakrophagen und (D) myeloiden DC in Flt3L vorbehandelten *S. pneumoniae*-infizierten Mäusen in An- und Abwesenheit von MC21 (Tag 3).

3.4 Effekt der Deletion von CCL2 auf den Verlauf und Schweregrad einer *Streptococcus pneumoniae* Infektion der Lunge

Ausgehend vom bisher erarbeiteten Konzept, dass dem Monozyten-rekrutierenden Chemokin CCL2 eine zentrale Funktion in der Mobilisierung mononukleärer Phagozyten im Verlauf der Pathogenese der *S. pneumoniae* Infektion der Lunge zukommt, wurden weiterführende Untersuchungen in Mäusen mit kongenitaler Deletion des CCL2 Gens (CCL2 KO Mäuse) durchgeführt. Diese Mäuse zeigen entsprechend bereits publizierter Daten unter Basalbedingungen einen normalen residenten Alveolarmakrophagenpool in der Lunge, weisen jedoch in verschiedenen anderen pulmonalen Infektionsmodellen (wie z.B. *Mycobacterium tuberculosis*) massiv aggravierte Infektionsverläufe mit konsekutiv gesteigerter Letalität auf [46, 47]. Analog zu den zuvor beschriebenen Experimenten wurden wiederum CCL2 KO Mäuse mit Serotyp 19 *S. pneumoniae* (EF3030) infiziert, welcher in Mäusen primär eine fokale Pneumonie verursacht, ohne Bakteriämie oder Sepsis zu induzieren [48-50]. Interessanterweise zeigte keine der *S. pneumoniae*-infizierten Wildtyp-Mäuse während des 14-tägigen Beobachtungszeitraums die Entwicklung einer Bakteriämie, wohingegen 50% der CCL2 KO Mäuse eine Bakteriämie nach *S. pneumoniae* Infektion entwickelten, zusammen mit einer im Vergleich zu Wildtyp-Mäusen signifikant erhöhten Letalität von ca. 55% an Tag 9 post Infektion (Abb. 16A und 16B; Abb. 1 in der Anlage 4). Passend zur gesteigerten Letalität ergaben weitere Untersuchungen, dass CCL2 KO Mäuse im Vergleich zu *S. pneumoniae*-infizierten Wildtyp-Mäusen eine signifikant schlechtere Erregerelimination aufwiesen (Abb. 16C und 16D; Abb. 1 in der Anlage 4). Zugleich zeigten CCL2 KO Mäuse parallel zur Bakteriämie ebenfalls eine im Vergleich zu *S. pneumoniae*-infizierten Wildtyp-Mäusen signifikant gesteigerte Lungenpermeabilität (Abb. 16E, Abb. 1 in der Anlage 4).



B

Tage	1	2	3	4	5	6	7	8	9	10	11	12	13	14
wt Bakterämie	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
KO Bakterämie	0/11	0/11	2/11	2/11	3/11	3/11	3/11	3/11	4/11	5/11	5/11	5/11	5/11	5/11

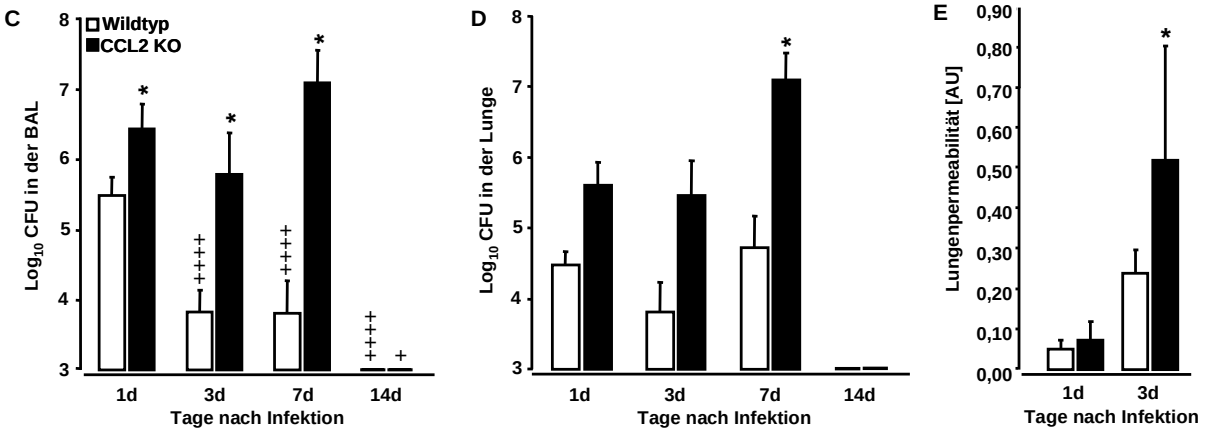


Abb. 16: Überleben, Bakteriämie und Erregerelimination in *S. pneumoniae*-infizierten Wildtyp-Mäusen und CCL2 KO Mäusen. (A) Überleben von CCL2 KO Mäusen (n=11) und Wildtyp-Mäusen (n=10). Die Tiere wurden intratracheal mit Serotyp 19 *S. pneumoniae* infiziert und über einen Zeitraum von 14 Tagen überwacht. (B) Kinetik der Bakteriämie-Entwicklung in *S. pneumoniae*-infizierten Wildtyp-Mäusen und CCL2 KO Mäusen. (C) Keimlast (CFU) in der BAL und (D) im Lungenparenchym *S. pneumoniae*-infizierter CCL2 KO Mäuse und Wildtyp-Mäuse. (E) Lungenpermeabilität in *S. pneumoniae*-infizierten CCL2 KO Mäusen und Wildtyp-Mäusen.

Weiterführende Untersuchungen zur Kinetik der entzündlichen Mobilisierung mononukleärer Phagozytensubsets in CCL2 KO Mäusen versus Wildtyp-Mäusen zeigten zu Beginn der Infektion sowohl in *S. pneumoniae*-infizierten CCL2 KO Mäusen als auch in Wildtyp-Mäusen eine Depletion des Alveolarmakrophagenpools, wobei die Re-Expansion des Alveolarmakrophagenpools in CCL2 KO Mäusen im Verlauf signifikant niedriger war als in Wildtyp-Mäusen nach Infektion (Abb. 17A; Abb. 4 in der Anlage 4). Wie Abbildung 17B zeigt, wiesen *S. pneumoniae*-infizierte

CCL2 KO Mäuse im Vergleich zu Wildtyp-Mäusen eine drastisch reduzierte *de novo* Rekrutierung alveolärer Exsudatmakrophagen auf (Abb. 17B, Abb. 4 in der Anlage 4).

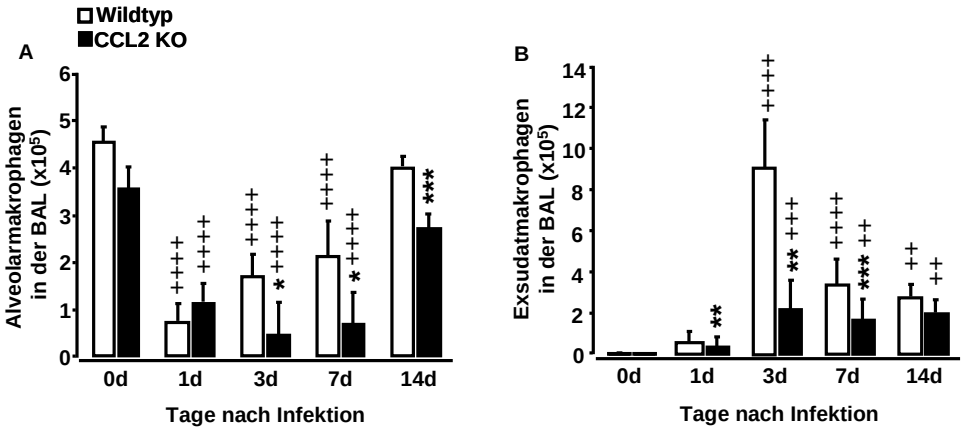


Abb. 17: Alveoläre Leukozytenrekrutierung in *S. pneumoniae*-infizierten CCL2 KO und Wildtyp-Mäusen. Alveolarmakrophagen (A) und Exsudatmakrophagen (B) der brochoalveolären Lavage *S. pneumoniae*-infizierter CCL2 KO Mäuse und Wildtyp-Mäuse wurden durchflusszytometrisch analysiert und quantifiziert. Für Details siehe Anlage 4.

4. Diskussion

4.1 Einfluss der downstream von CCR2 agierenden Phosphatidylinositol 3-Kinase γ (PI3Kγ) auf die entzündliche Monozytenrekrutierung sowie die Reparaturphase im Verlauf der Pneumokokken-Pneumonie der Maus

In den vorliegenden Untersuchungen zur Funktion der PI3Kγ in der *S. pneumoniae*-induzierten Lungeninfektion konnte gezeigt werden, dass sowohl die kongenitale Defizienz von PI3Kγ als auch die Inhibition von PI3Kγ mittels spezifischer „small-molecule inhibitors“ (AS-605240) zu einer reduzierten Exsudatmakrophagenrekrutierung im Verlauf der Pneumokokkeninfektion führte. Parallel hierzu führte die Blockade bzw. die Inhibition von PI3Kγ ebenso zu einer massiv gestörten Reparaturphase, was darauf hindeutet, dass entzündlich rekrutierte Exsudatmakrophagen an der Wiederherstellung der Lungenhomöostase im Verlauf einer Pneumokokken-Pneumonie beteiligt sind. In diesem Zusammenhang konnte eine andere Studie zeigen, dass die Depletion des Alveolarmakrophagenpools mittels intratrachealer Applikation von liposomalem Clodronat zu Beginn einer Pneumokokkeninfektion die Reparaturphase im Verlauf einer Pneumokokken-Pneumonie entscheidend verschlechterte [51]. Diese Daten zeigen, dass Makrophagen der Lunge eine wichtige Funktion in der Wiederherstellung der Lungenhomöostase haben.

In der vorliegenden Arbeit zeigten *S. pneumoniae*-infizierte PI3Kγ KO Mäuse eine Neutrophilenrekrutierung vergleichbar der von *S. pneumoniae*-infizierten Wildtyp-Mäusen. Gleichzeitig zeigten PI3Kγ-defiziente Mäuse dennoch eine signifikant höhere Keimlast in der bronchoalveolären Lavageflüssigkeit im Vergleich zu Wildtyp-Mäusen nach Infektion mit *S. pneumoniae*. Diese reduzierte Erregerelimination in PI3Kγ KO Mäusen ist wahrscheinlich auf eine verminderte Produktion von Sauerstoffradikalen (respiratorischer Burst) in neutrophilen Granulozyten zurückzuführen, da bekannt ist, dass PI3Kγ Defizienz eine attenuierte Burst-Induktion in neutrophilen Granulozyten bedingt. Im Modell der septischen Peritonitis konnte Hirsch et al. zeigen, dass PI3Kγ KO Mäuse neben einer verminderten Makrophagenrekrutierung ebenso signifikant weniger neutrophile Granulozyten mobilisierten, welche zudem einen reduzierten respiratorischen Burst nach Stimulation mit fMLP *in vitro* aufwiesen [29]. Vor diesem Hintergrund deuten unsere

Daten darauf hin, dass es wahrscheinlich auch unter den Bedingungen einer Pneumokokken-Pneumonie zu keiner vollständigen Burst-induktion in rekrutierten neutrophilen Granulozyten von PI3Kγ KO Mäusen oder Inhibitor-vorbehandelten Wildtyp-Mäusen kommt, was demzufolge einen negativen Einfluss auf die Wirtsabwehr gegenüber inhalierten bakteriellen Erregern mit konsekutiv gestörter Keimelimination hat. Somit zeigen die in der vorliegenden Arbeit vorgestellten Untersuchungen zur Funktion von PI3Kγ in der Pneumokokken-Pneumonie der Maus, dass PI3Kγ Defizienz zu einer massiven Störung der Exsudatmakrophagenmobilisierung, nicht jedoch der Neutrophilenrekrutierung beiträgt und aller Wahrscheinlichkeit nach auch die über respiratorschen Burst vermittelte Abwehrfunktion rekrutierter neutrophiler Granulozyten verändert. Dies führt im Zusammenspiel schlussendlich zu einer Störung der Erregerelimination im bronchoalveolären Kompartiment sowie zu einer attenuierten Heilungs- und Reparaturphase im späteren Verlauf der Pneumokokken-Pneumonie und bedingt zudem die signifikant gesteigerte Letalität der PI3Kγ KO Mäuse im Vergleich zu Wildtyp-Mäusen. Interessanterweise konnten andere Arbeitsgruppen zeigen, dass PI3Kγ Defizienz bzw. Applikation von PI3Kγ-spezifischen Inhibitoren in einem Modell der Kollagen-induzierten Arthritis der Maus die Mobilisierung neutrophiler Granulozyten in das entzündete Gelenk attenuieren konnte, welches zugleich eine Verbesserung der klinischen Symptomatik bedingte [37]. Im Kontext mit den in der vorliegenden Arbeit präsentierten Daten kann gegenwärtig jedoch nicht ausgeschlossen werden, dass die pharmakologische Applikation von PI3Kγ-spezifischen Inhibitoren zur Therapie chronisch-entzündlicher Erkrankungen (wie z.B. rheumatoide Arthritis) potentiell auch unerwünschte Nebeneffekte auf die Wirtsabwehrfunktion der Lunge gegenüber inhalierten bakteriellen Erregern hat [52].

4.2 Verstärkung der Wirtsabwehr der Lunge gegenüber einer *S. pneumoniae* Infektion durch konnatale Überexpression des Monozyten rekrutierenden Chemokins CCL2 in der Lunge

Basierend auf den zuvor beschriebenen Erkenntnissen, dass die Deletion oder spezifische Inhibition von PI3Kγ zu einer Attenuierung der entzündlichen Makrophagenmobilisierung in die Lunge mit konsekutiv aggraviertem Verlauf der Pneumokokken-Pneumonie führte, postulierten wir, dass eine verstärkte

Mobilisierung mononukleärer Phagozyten-Subsets in die Lunge den Verlauf und Schweregrad einer *S. pneumoniae*-induzierten Lungeninfektion verbessert. Zur Überprüfung dieser Hypothese wurde ein Mausmodell verwendet, in dem das humane Monozyten rekrutierende Chemokin CCL2 in Typ II Alveolarepithelzellen konstitutiv überexprimiert wird und es daraufhin zu einer verstärkten Akkumulation monozytärer Zellen im alveolären Kompartiment und Lungenparenchym kommt [42]. Wir konnten sehr klar zeigen, dass die Infektion von CCL2 transgenen Mäusen im Vergleich zu Wildtyp-Mäusen mit *S. pneumoniae* zu einer signifikant verbesserten Erregerelimination sowie geringeren Letalität führte. Diese Daten zeigen erstmals, dass ein konstitutiv expandierter mononukleärer Phagozytenpool der Lunge die Wirtsabwehrfunktion der Lunge gegenüber inhalierten bakteriellen Erregern wie *S. pneumoniae* signifikant verbessern kann und zu einem gesteigerten Überleben der Mäuse führt. Auf der anderen Seite entwickelten CCL2 transgene Mäuse nach Infektion mit *S. pneumoniae* jedoch auch transiente fibroproliferative Umbauprozesse entsprechend einer Bronchiolitis obliterans. Interessanterweise führte die Applikation eines spezifischen Antikörpers gegen den Rezeptor CCR2 (MC21) zu einer vollständigen Remission der Bronchiolitis obliterans, gleichzeitig aber auch zu einer massiv gestörten Reparaturphase im Verlauf der Pneumokokken-Pneumonie. Auch hier ging die Störung der Reparaturphase mit einer attenuierten Rekrutierung von Exsudatmakrophagen und einem erneuten Auswachsen der Erreger im pulmonalen Kompartiment einher, wodurch erneut das Konzept unterstützt wird, dass inflammatorisch rekrutierte Exsudatmakrophagen zentral an der Auflösung konsolidierter Infiltrate in der Lunge nach *S. pneumoniae* Infektion beteiligt sind. Diese Befunde lassen den Schluss zu, dass Exsudatmakrophagen zumindest indirekt einen wichtigen Beitrag zu einer suffizienten Erregerelimination in der Lunge leisten (siehe Anlage 1 und 2). Die Daten der vorliegenden Arbeit belegen darüber hinaus, dass die Interaktion des Chemokins CCL2 mit seinem Rezeptor CCR2 eine zentrale Rolle in der Wirtsabwehr der Lunge gegenüber *S. pneumoniae* spielt und möglicherweise auch an der Regulation derjenigen Mechanismen beteiligt ist, welche zu fibroproliferativen Umbauprozessen in der Lunge unter akuten Entzündungsbedingungen führen. Untersuchungen anderer Arbeitsgruppen konnten in diesem Zusammenhang zeigen, dass murine Fibroblasten über CCR2-abhängige Pathways in die Lunge rekrutiert werden und durch CCL2 zu einer verstärkten Sekretion von Kollagen stimuliert werden können [53]. Diese Daten könnten

möglicherweise darauf hindeuten, dass eine verstärkte Freisetzung von CCL2 und konsekutiv verstärkte Mobilisierung CCR2-positiver leukozytärer und/oder mesenchymaler Zellpopulationen in das pulmonale Kompartiment in Anwesenheit von Entzündung fibroproliferative Umbauprozesse in der Lunge begünstigt. Der akuten Entzündung scheint hierbei eine ‚Trigger'-funktion zuzukommen, da unbehandelte CCL2 transgene Mäuse trotz expandierter pulmonaler mononukleärer Phagozytensubsets sowie stark erhöhter CCL2 Spiegel in der BAL keine spontanen fibroproliferativen Umbauprozesse entwickelten (Anlage 2). Allerdings ist bis dato nicht klar, welcher Entzündungsstimulus oder aber welche entzündlich rekrutierte leukozytäre bzw. mesenchymale Zellpopulation die beobachtete Bronchiolitis obliterans in CCL2 transgenen Mäusen nach Infektion mit *S. pneumoniae* auslöste. Weiterführende Untersuchungen sind zur Klärung dieser Fragestellung erforderlich.

4.3 Effekt der pulmonalen Expansion dendritischer Zellen auf den Verlauf einer *Streptococcus pneumoniae* induzierten Pneumonie

Basierend auf den zuvor beschriebenen Befunden, dass eine konstitutive Überexpression von CCL2 in der Lunge zu einer Verbesserung der Infektabwehr gegenüber *S. pneumoniae* führte, stellte sich im weiteren Verlauf der vorliegenden Arbeit die Frage, welchen Effekt eine selektiv verstärkte Mobilisierung individueller mononukleärer Phagozyten-Subsets in die Lunge auf den Verlauf der Pneumokokken-Pneumonie hat. Insbesondere dendritische Zellen der Lunge lagen im Fokus unseres Interesses, da sie strategisch zwischen Alveolarepithel und Kapillarendothel des Lungeninterstitiums lokalisiert sind und über ihre Zellausläufer luminales Antigen aufnehmen können und somit potentiell auch einen positiven Beitrag zur Elimination inhalierter bakterieller Erreger leisten [54, 55]. Daher behandelten wir Wildtyp-Mäuse 9 Tage lang mit dem DC-spezifischen Wachstumsfaktor Fms-like tyrosine kinase 3 ligand (Flt3L), was zu einer massiven Expansion des DC-Pools der Lunge sowie zur transienten Monozytose im peripheren Blut führte. Wir konnten zeigen, dass Flt3L-vorbehandelte, *S. pneumoniae*-infizierte Mäuse im Vergleich zu Kontrollmäusen gemessen an ihrer schlechteren Erregerelimination, gesteigerten Vaskulitis und Lungenpermeabilität sowie reduzierten Überleben keinen Vorteil in der Wirtsabwehr von *S. pneumoniae* hatten. Betrachtet man jedoch, dass weder Lungenpermeabilitätserhöhung noch Vaskulitis in

Flt3L vorbehandelten Mäusen in Abwesenheit von *S. pneumoniae* zu beobachten waren, lässt sich daraus ableiten, dass ein entzündlich ausgelöster Mechanismus im Zusammenspiel mit der verstärkten DC-Akkumulation in der Lunge zu einer Entgleisung der pulmonalen Entzündungsprozesse führte und das Überleben Flt3L vorbehandelter, *S. pneumoniae*-infizierter Mäuse verschlechterte. Möglicherweise spielt in diesem Zusammenhang gerade die beobachtete erhöhte Lungenpermeabilität bei vorexpandiertem pulmonalem DC-Pool eine entscheidende Rolle. Da in einer weiteren Untersuchung zur Pathobiologie pulmonaler DC im Kontext bakterieller Infektionen der Lunge auch die Applikation von *E. coli* Endotoxin bzw. *Klebsiella pneumoniae* zu einer konsekutiv gesteigerten Lungenpermeabilität führte [56], scheint der hier zugrunde liegende Pathomechanismus unabhängig von der verwendeten Bakterien-Spezies zu sein. Wenngleich bis dato zumeist neutrophile Granulozyten als diejenigen Entzündungszellen verantwortlich gemacht wurden, welche eine Störung der pulmonalen Schrankenfunktion bei Inflammation verursachen können [57], so entwickeln auch neutropene Patienten als häufige Komplikation neben ihrer Infektabwehrschwäche ein akutes Atemnotsyndrom (ARDS), welches unter anderem auf einer gestörten Schrankenfunktion basiert. Möglicherweise deuten die vorliegenden Daten erstmals darauf hin, dass dendritische Zellen der Lunge, welche sowohl mit Alveolarepithelzellen als auch mit Kapillarendothelzellen in unmittelbarem Kontakt stehen, aufgrund ihres hohen proinflammatorischen Potentials unter Infektionsbedingungen zur pulmonalen Schrankenstörung beitragen. Mit den vorab gezeigten Befunden zur DC Pathobiologie in der Pneumokokken-Pneumonie konnten wir jedoch unsere Hypothese nicht verifizieren, dass ein maximal expandierter pulmonaler DC-Pool zur Infektabwehr bakterieller Infektionen beiträgt. Dennoch müssen weiterführende Untersuchungen klären, ob nicht etwa submaximale Gaben an Flt3L für eine abgestufte Akkumulation von DC im Lungenparenchym die Infektabwehr der Mauslunge gegenüber *S. pneumoniae* verbessern könnten.

4.4 Effekt der Deletion von CCL2 auf den Verlauf und Schweregrad einer *Streptococcus pneumoniae* Infektion der Lunge

Die in den Anlagen 1-3 vorgestellten Untersuchungen zeigten klar, dass die CCL2-CCR2 Achse eine zentrale Bedeutung für die entzündliche

Makrophagenmobilisierung im Kontext pulmonaler Infektionen mit *S. pneumoniae* spielt. Demgegenüber zeigte eine kürzlich von Dessing et al. veröffentlichte Arbeit, dass sich für CCL2 im Modell der Pneumokokken-Pneumonie der Maus bei Infektion mit einem invasiv wachsenden *S. pneumoniae* Serotyp 3 kein protektiver Effekt in der Pathogenese der Pneumokokken-Pneumonie nachweisen lies [58]. Bei der Bewertung jener von unseren Daten abweichenden Ergebnisse müssen jedoch mehrere Aspekte berücksichtigt werden: Zunächst wurden in der Studie von Dessing et al. im Gegensatz zu unserer Studie (BALB/c) C57BL/6 Wildtyp-Mäuse verwendet. Daneben lassen sich auch hinsichtlich der Methode der Pneumokokkeninfektion klare Unterschiede zwischen der Untersuchung von Dessing et al. und unseren Untersuchungen feststellen. Dessing et al. infizierten ihre Versuchsmäuse via intranasaler Applikation der Bakterien, während die in der vorliegenden Arbeit beschriebenen Experimente (Anlage 1-3) alle via intratrachealer Infektion durchgeführt wurden. Eine intranasale Applikation von Pneumokokken kann sowohl zu Sinusitis, Otitis media als auch zur Entwicklung septischer Verlaufsformen, wie z.B. einer septischen Meningitis führen. Demgegenüber induziert man bei intratrachealer Applikation von Pneumokokken ganz primär eine Pneumonie und, in Abhängigkeit vom verwendeten Serotyp, keine invasiven Krankheitsverläufe. Diesbezüglich besteht jedoch einer der zentralen Unterschiede zwischen den beiden Untersuchungen, da Dessing et al. einen hochvirulenten Serotyp 3 *S. pneumoniae* verwendeten, welcher bekanntermaßen sehr rasch eine Bakteriämie und Sepsis in Mäusen induziert. Berücksichtigt man hierbei, mit welcher Kinetik sich ein Chemokingradient zwischen dem pulmonalen Kapillarbett und dem Alveolarraum der Lunge etabliert und welche Zeitspanne erforderlich ist, um unter entzündlichen Bedingungen inflammatorische Monozyten und daraus abgeleitet Exsudatmakrophagen in die Lunge zu rekrutieren, so fällt auf, dass es sehr wohl möglich ist, das Bakteriämie und Sepsis bei Verwendung eines virulenten Pneumokokkenstammes bereits etabliert sind, bevor sich ein effektiver Chemokin-getriebener Makrophageninflux in den Alveolarraum etablieren konnte. Vor diesem Hintergrund kommen wir zu dem Schluss, dass aller Wahrscheinlichkeit nach Chemokin-getriebene Rekrutierungsprozesse von Exsudatmakrophagen in die Lunge bei bakteriellen Infektionen ihren größtmöglichen Nutzen für den Wirt bezüglich bakterieller Infektabwehr dann aufweisen sollten, wenn die Lunge mit nicht invasiv wachsenden Pneumokokken infiziert wurde. Zur Überprüfung dieser Hypothese

wurden in der vorliegenden Arbeit analog zu den Untersuchungen von Dessing et al. CCL2 KO Mäuse und Wildtyp-Mäuse im C57BL/6 background mit einem *per se* nicht invasiven Serotyp 19 *S. pneumoniae* intratracheal infiziert. Dieser Pneumokokkenstamm induziert in Mäusen primär eine fokale Pneumonie, entsprechend einer Lobärpneumonie beim Menschen, ohne dass es zur Entwicklung von Bakteriämie und Sepsis oder zu einer gesteigerten Letalität kommt [48-50]. Interessanterweise konnten wir zeigen, dass mit Serotyp 19 *S. pneumoniae* infizierte CCL2 KO Mäuse im Vergleich zu Wildtyp-Mäusen bereits ab Tag 3 nach Infektion eine Bakteriämie entwickelten, welche sich schlussendlich bei ca. 50% der infizierten CCL2 KO Mäuse nachweisen ließ. Demgegenüber konnte bei keiner der *S. pneumoniae*-infizierten Wildtyp-Mäuse innerhalb des 14-tägigen Beobachtungszeitraumes die Entwicklung einer Bakteriämie beobachtet werden. Einhergehend mit diesem aggravierten Infektionsverlauf zeigten CCL2-defiziente Mäuse im Vergleich zu Wildtyp-Mäusen eine signifikant reduzierte Mobilisierung mononukleärer Phagozyten in die Lunge, welches zu einer signifikant gesteigerten Letalität in CCL2 KO Mäusen führte. Diese Daten lassen sich im Kontext mit den bereits vorab beschriebenen Befunden in PI3Kγ defizienten Mäusen und CCL2-transgenen Mäusen dahingehend interpretieren, dass die Unterbrechung der CCL2-CCR2 Achse für den Verlauf und Schweregrad einer bakteriellen Infektion der Lunge und damit für die Wirtsabwehr von fataler Konsequenz ist. Unseren Untersuchungen zufolge kann die Lunge aufgrund der CCL2-abhängigen Exsudatmakrophagenmobilisierung eine nicht-invasive Pneumokokkeninfektion auf das pulmonale Kompartiment beschränken und damit die Entwicklung einer invasiven Erkrankung verhindern. Demzufolge ist die Virulenz eines inhalierten Erregers nicht nur durch sein Arsenal an Virulenzfaktoren, sondern auch durch die Infektabwehrkapazität der Lunge definiert.

Kumulativ bilden die vorliegenden Arbeiten die Grundlage zur Formulierung des Konzeptes, dass zukünftige Antibiotika-unabhängige Behandlungsstrategien als einen wesentlichen Baustein zur Verbesserung der Infektabwehr der Lunge gegenüber inhalierten bakteriellen Erregern eine verstärkte pulmonale Mobilisierung und/ oder Aktivierung inflammatorisch rekrutierter Exsudatmakrophagen zum Ziel haben sollten.

5. Ausblick

In der vorliegenden Arbeit wurden neue Aspekte zur Bedeutung des mononukleär-phagozytären Systems in der pulmonalen Infektabwehr inhalierter bakterieller Erreger am Beispiel der *S. pneumoniae*-induzierten Lungeninfektion der Maus erarbeitet. Zugleich bieten die Befunde der vorliegenden Arbeit mehrere Ansatzmöglichkeiten für weiterführende Untersuchungen. Insbesondere zwei Aspekte scheinen von besonderer Relevanz zu sein: Zum einen konnten wir zeigen, dass die konnatale Überexpression von CCL2 den Schweregrad und Verlauf einer Pneumokokken-Pneumonie positiv beeinflusste. Demgegenüber ist aber nicht klar, ob nicht möglicherweise auch eine transiente anstelle einer konnatalen pulmonalen Überexpression von CCL2 die Infektabwehr gegenüber *S. pneumoniae* verbessert. Gerade aber solche Interventionsmassnahmen könnten vielversprechende und möglicherweise sogar therapeutisch nutzbare Schritte im Kampf gegen Infektionskrankheiten der Lunge repräsentieren und sollen das Ziel weiterführender Untersuchungen sein. Zum zweiten konnten wir zeigen, dass entgegen unseren Erwartungen die verstärkte Mobilisierung pulmonaler myeloider dendritischer Zellen in die Lunge von Mäusen keinen Vorteil in der Infektabwehr der Lunge erbrachte. Demgegenüber steht jedoch, dass dendritische Zellen der Lunge sehr effektiv inhaliertes Antigen aufnehmen und intrazellulär prozessieren können, so dass möglicherweise eine abgestufte Mobilisierung dendritischer Zellen anstelle der in der vorliegenden Arbeit demonstrierten maximalen Akkumulation von dendritischen Zellen im Lungeninterstitium die Infektabwehrlage der Lunge gegenüber inhalierten bakteriellen Erregern doch verbessern könnte. Zukünftige experimentelle Konzepte basieren daher auf einer graduell zunehmenden Applikationsdauer des Wachstumsfaktors Flt3L, um auf diese Weise eine abgestufte Rekrutierung von pulmonalen DC in der Lunge zu erwirken. Diese Arbeiten sollten einen genaueren Eindruck über das Potential dendritischer Zellen als therapeutisches Mittel im Kampf gegen Pneumokokken-Pneumonien ermöglichen. Auch hier gilt wie in der vorab beschriebenen Perspektive einer transienten CCL2 Überexpression in der Lunge, dass die geplanten weiterführenden Untersuchungen ein genaueres Urteil über die Relevanz der vorgestellten „zelltherapeutischen“ Verfahren zur Verbesserung der Infektabwehr der Lunge gegenüber *S. pneumoniae* erlauben werden.

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8. Curriculum vitae

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Publikationsliste

1. Maus, U.A., M. Backi, **C. Winter**, M. Srivastava, M. K. Schwarz, T. Rückle, J. C. Paton, D. Briles, M. Mack, T. Welte, R. Maus, R. M. Bohle, W. Seeger, C. Rommel, E. Hirsch, J. Lohmeyer, K. T. Preissner.
Importance of Phosphoinositide 3-Kinase γ in the host defense against pneumococcal infection.
Am J Respir Crit Care Med 2007; 175: 958-966

2. **Winter**, **C.**, K. Taut, M. Srivastava, F. Länger, M. Mack, D. E. Briles, J. C. Paton, R. Maus, T. Welte, M. D. Gunn, U. A. Maus.
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.
J Immunol 2007; 178: 5828-5838

3. **Winter, C.**, K. Taut, F. Länger, M. Mack, D. E. Briles, J. C. Paton, R. Maus, M. Srivastava, T. Welte, U. A. Maus.
FMS-like tyrosine kinase 3 ligand aggravates the lung inflammatory response to *Streptococcus pneumoniae* infection in mice: role of dendritic cells.
J Immunol 2007; 179: 3099-3108

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Lack of lung mononuclear phagocyte recruitment elicits sepsis in mice infected with non-invasive *Streptococcus pneumoniae*. (Manuskript zur Publikation eingereicht)

9. Anlagenverzeichnis

Anlage 1 Maus, U.A., M. Backi, **C. Winter**, M. Srivastava, M. K. Schwarz, T. Rückle, J. C. Paton, D. Briles, M. Mack, T. Welte, R. Maus, R. M. Bohle, W. Seeger, C. Rommel, E. Hirsch, J. Lohmeyer, K. T.Preissner. Importance of Phosphoinositide 3-Kinase γ in the host defense against pneumococcal infection. *Am. J. Respir. Crit. Care Med.* 2007; 175: 958-966.

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Importance of Phosphoinositide 3-Kinase γ in the Host Defense against Pneumococcal Infection

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***Rationale*: The pivotal role of phosphoinositide 3-kinase γ (PI3Kγ) in leukocyte recruitment makes it an attractive target for immunomodulatory therapy. However, interfering with PI3Kγ signaling might increase the risk of bacterial infections in humans.**

***Objectives*: We hypothesized that deletion or pharmacologic inhibition of PI3Kγ would impair the lung inflammatory response to the prototypic gram-positive bacterial pathogen *Streptococcus pneumoniae*.**

***Methods*: PI3Kγ knockout (KO) and wild-type mice were infected with *S. pneumoniae* or challenged with the pneumococcal virulence factor pneumolysin (PLY), and inflammatory leukocyte recruitment, bacterial pathogen elimination, and resolution/repair processes were determined.**

***Measurements and Main Results*: PI3Kγ KO mice challenged with PLY responded with lung edema and neutrophilic alveolitis, but showed a drop in alveolar macrophages and failed to recruit exudate macrophages when compared with wild-type mice. *S. pneumoniae*–infected PI3Kγ KO mice and wild-type mice pretreated with the pharmacologic inhibitor AS-605240 recruited similar numbers of neutrophils but substantially fewer exudate macrophages into their lungs than control animals. They also displayed a significantly reduced lung pneumococcal clearance and showed an impaired resolution/repair process, leading to progressive pneumococcal pneumonia.**

***Conclusions*: PI3Kγ gene deletion or pharmacologic inhibition of PI3Kγ leads to perturbations of critical innate immune responses of the lung to challenge with *S. pneumoniae*. These data are of clinical relevance for the treatment of chronic inflammatory diseases where pharmacologic inhibition of PI3Kγ signaling to attenuate effector cell recruitment may have implications for innate immune surveillance of remote organ systems.**

Keywords: lung; infection; macrophage

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

No data are currently available addressing the role of phosphoinositide 3-kinase γ (PI3Kγ) deletion or inhibition on the lung host defense to *Streptococcus pneumoniae* infection.

What This Study Adds to the Field

Blockade of PI3Kγ activity suppresses effector cell recruitment, reduces lung pneumococcal clearance, and may have implications for the immune surveillance of distant organ systems.

Directional leukocyte recruitment toward inflamed tissues was recently shown to be largely dependent on phosphoinositide 3-kinase isoform γ (PI3Kγ) (1–3), which belongs to the family of class I PI3 kinases, making PI3Kγ an attractive target for immunomodulatory therapy (4). All class I PI3Ks are heterodimeric proteins composed of a 110-kD catalytic subunit (class IA: p110α, p110β, and p110δ; class IB: p110γ) and an 85-kD (p85) or a 101-kD (p101) regulatory subunit, respectively. Although the PI3Kα and PI3Kβ isoforms are ubiquitously expressed, the PI3Kδ and PI3Kγ isoforms are largely confined to leukocytes. PI3Kγ activity is regulated by the p101 regulatory adapter associated with seven-transmembrane G protein–coupled receptors (GPCRs). Activation of PI3Kγ links the chemokine-induced GPCR activation to the generation of phosphatidylinositol 3,4,5-triphosphates (PtdIns $[3–5]P_3$), which play an essential role in downstream signaling to elicit directional cell movement (2, 5). Consequently, PI3Kγ-deficient leukocytes demonstrated impaired migration to various chemoattractant stimuli, which was found to be more confined to the mononuclear phagocytic as compared with the neutrophilic migratory response in a mouse model of septic peritonitis (2, 6, 7). Moreover, PI3Kγ-deficient neutrophils demonstrated reduced respiratory burst formation in response to GPCR-mediated agonist activation *in vitro* (2). In addition, most recently developed, highly specific, orally active, small-molecule PI3Kγ inhibitors were shown to be effective in suppressing joint inflammation in mouse models of rheumatoid arthritis (8).

Streptococcus pneumoniae is the most prevalent gram-positive bacterium causing community-acquired pneumonia, septic meningitis, and otitis media worldwide. Despite its considerable clinical importance, the molecular mechanisms by which *S. pneumoniae* overcomes innate immune responses are only partially understood. The pathogenicity of *S. pneumoniae* appears to be largely defined by its ability to generate a variety of virulence factors, among which the pneumococcal virulence factor pneumolysin

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

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(PLY) is considered to be of major importance. PLY is an intracellular 53-kD protein and is released during pneumococcal autolysis (9). It belongs to the family of cholesterol-binding cytolysins and is highly cytotoxic toward target cells due to its pore-forming activities. In addition, PLY has been reported to act as a pathogen-associated molecular pattern by signaling via Toll-like receptor 4 (TLR4) and to induce TLR4-dependent apoptosis in macrophages with protective effects in pneumococcal pathogenesis (10, 11).

Recent reports from our group demonstrated that intratracheal application of PLY into the lungs of mice not only induced severe lung edema but also provoked a drastic depletion of the resident alveolar macrophage pool within the lungs, implying that PLY not only induces cytotoxicity toward lung epithelial cells (12, 13) but also promotes colonization of the lower respiratory tract by exerting strong cytotoxicity toward sentinel host defense cells of the lung (14). Consequently, to overcome the PLY-induced loss of alveolar macrophage function, the host must rapidly mount an inflammatory leukocyte influx to purge pneumococcal infection from distal airspaces (14).

The blockade of PI3Kγ by small-molecule inhibitors may prove to be a valid approach to modulate excessive effector leukocyte accumulations in inflamed tissues, where leukocyte recruitment is correlated with disease progression, as occurs in rheumatoid arthritis and other diseases (8, 15–17). On the other hand, interfering with inflammatory leukocyte trafficking may adversely affect protective innate immunity of other remote organ systems, such as the lung, thereby increasing the risk of bacterial infections in humans. The current study was performed to test the hypothesis that PI3Kγ gene deletion or pharmacologic inhibition of PI3Kγ signaling would adversely affect the lung innate host defense to challenge with the prototypic gram-positive bacterial pathogen *S. pneumoniae*.

METHODS

Animals

Wild-type and PI3Kγ knockout (KO) mice (weight, 20–25 g; age, 8–12 wk) were generated on a 129/Sv background as described previously (2) and maintained under specific pathogen–free conditions. All animal experiments were approved by our local government authorities (LAVES, Oldenburg, Germany). Experiments were performed under blinded conditions.

Reagents

Native PLY was purified from recombinant *Escherichia coli* as described previously (18). Recombinant murine CCL2 protein (monocyte chemotactic protein-1) was obtained from Peprotech (Offenbach, Germany). The PI3Kγ-specific inhibitor AS-605240 was used as described recently (8). Rat anti-mouse anti-CCR2 monoclonal antibody MC21 was used as described recently (16, 19). For further details, *see* the online supplement.

Treatment of Animals with PLY or CCL2

Intratracheal application of PLY or CCL2 was performed as described recently (14) and is further outlined in the online supplement.

Bronchoalveolar Lavage

Collection of serum and bronchoalveolar lavage (BAL) fluid for the isolation and quantification of resident alveolar macrophages and alveolar recruited leukocytes was performed as described recently (16, 20) and is outlined in the online supplement.

Isolation of Bone Marrow Cells and Transplantation into Recipient Animals

Bone marrow cells were isolated under sterile conditions from the tibias and femurs of sex-matched, syngeneic PI3Kγ KO mice, and transplanted into lethally irradiated wild-type mice following recently described protocols (16, 21) (*see* the online supplement).

Preparation of Mouse Bone Marrow–derived Monocytes and *In Vitro* Chemotaxis

Preparation of mouse bone marrow–derived monocytes (BMDMs) and *in vitro* chemotaxis assays with murine BMDMs was performed as recently described (5) and is further outlined in the online supplement.

Culture and Quantification of *S. pneumoniae* and Infection in PI3Kγ KO and Wild-Type Mice

For infection experiments, we used the PLY-producing clinical isolate of *S. pneumoniae* capsular group 19 strain EF3030 ($\sim 4 \times 10^7$ cfu/mouse), and for survival experiments, the capsular group 2 strain D39 ($\sim 2 \times 10^3$ cfu/mouse), both of which were grown in Todd-Hewitt broth (Difco, Heidelberg, Germany) supplemented with 0.1% yeast extract to midlog phase (22), as outlined in the online supplement in detail.

Lung Histopathology and Lung Permeability Measurements

Lung histopathology and lung permeability were analyzed as described in the online supplement.

Pharmacologic Blockade of PI3Kγ in *S. pneumoniae*–infected Wild-Type Mice

We evaluated the effect of pharmacologic blockade of PI3Kγ on the pneumococcal clearance capacity of wild-type mice pretreated with the PI3Kγ-specific inhibitor AS-605240. Briefly, AS-605240 compound was dissolved in vehicle (0.5% carboxymethylcellulose/0.25% Tween-20), as recently described (8), and injected intraperitoneally in wild-type mice 2 hours before and at 12-hour intervals subsequent to infection in the mice with *S. pneumoniae* (EF3030, 2×10^7 cfu/mouse) at a dose of 30 mg/kg body weight.

Determination of Bacterial Loads in the Lungs of PI3Kγ KO and Wild-Type Mice

Bacterial loads in the lungs of *S. pneumoniae*–infected PI3Kγ KO and wild-type mice were calculated from whole lung washes, as described recently (22), and are outlined in the online supplement.

Statistics

All data are given as mean ± SD. Differences between control animals and respective treatment groups over time were analyzed by analysis of variance 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 (Version 14.0; SPSS, Inc., Chicago, IL). Survival curves were compared by log-rank test. Statistically significant differences among various treatment groups were assumed when p values were less than 0.05.

RESULTS

Lung Inflammatory Response of Wild-Type and PI3Kγ KO Mice Challenged with PLY

Intratracheal application of PLY induced a rapid, progressive increase in lung permeability in both wild-type and PI3Kγ KO mice, peaking at 6 hours post-treatment and declining thereafter (Figure 1A). In line with this finding, histopathologic examination of lung tissues collected from PLY-challenged PI3Kγ KO and wild-type mice at 6 and 24 hours post-treatment showed focally accentuated intraalveolar edema in mice of both treatment groups (data not shown). In addition, intratracheal administration of PLY into the lungs of wild-type and PI3Kγ KO mice elicited the alveolar accumulation of neutrophils, with significantly increased numbers noted at 12 hours up until 48 hours post-treatment. However, no significant differences were observed between groups, demonstrating that neutrophils do not require PI3Kγ activity to accumulate within the alveolar airspace of PLY-challenged mice (Figure 1B). Importantly, instillation

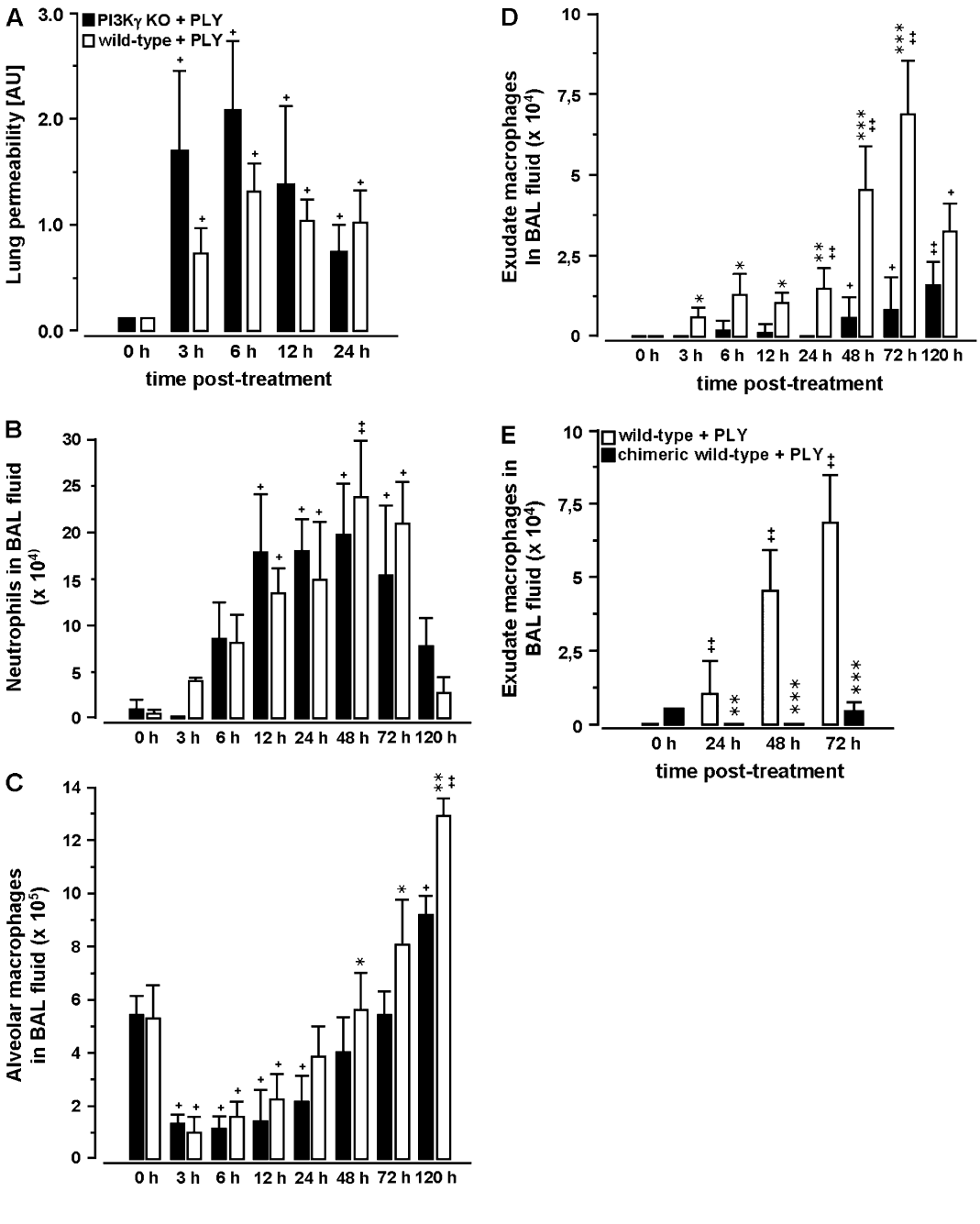

Figure 1. Effect of intratracheal pneumolysin (PLY) application on the lung inflammatory response in phosphoinositide 3-kinase γ (PI3Kγ) knockout (KO) versus wild-type mice. PI3Kγ KO mice and wild-type mice (*A–D*) or, in selected experiments, chimeric wild-type mice (*E*) received intratracheal applications of PLY (40 Hounsfield units per mouse), as indicated. Control mice (0 h time points) received vehicle (phosphate-buffered saline/0.1% human serum albumin) only for 24 hours. (*A*) One hour before being killed, mice received intravenous injections of fluorescein isothiocyanate (FITC)–labeled albumin. At the indicated time points, mice were killed and lung permeability changes were determined by measuring FITC-albumin leakage into the lungs. (*B–D*) Total numbers of neutrophils (*B*), alveolar macrophages (*C*), and exudate macrophages (*D*) quantified from bronchoalveolar lavage (BAL) fluid specimens of mice challenged with PLY for the indicated time points. (*E*) In selected experiments, the effect of peripheral blood leukocyte–restricted elimination of PI3Kγ on the accumulation of exudate macrophages in response to PLY was analyzed. Wild-type mice and chimeric wild-type mice with a PI3Kγ-deficient hematopoietic system were challenged intratracheally with PLY. At the indicated time points, mice were killed and numbers of alveolar recruited exudate macrophages were determined. Values are shown as mean ± SD of five mice per group and time point (6 h time point, n = 9). $^{+}$Significant ($p < 0.05$) increase/decrease compared with the respective 0 h control values; ‡Significant ($p < 0.01$) increase/decrease compared with the respective 0 h control values; *Single*, *double stacked*, and *triple stacked asterisks* indicate significant difference between wild-type and PI3Kγ KO or chimeric wild-type mice: $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

of PLY into the lungs of wild-type mice and PI3Kγ KO mice induced a strong depletion of resident alveolar macrophages, with a maximal drop observed between 3 and 12 hours post-treatment (Figure 1C). Of note, compensatory alveolar exudate macrophage recruitment to restore alveolar macrophage homeostasis was primarily observed in wild-type mice in response to PLY, and was significantly impaired in PI3Kγ KO mice (Figure 1D). Consequently, restoration of the alveolar macrophage pool after PLY challenge was achieved earlier in wild-type mice as compared with PI3Kγ KO mice (Figures 1C and 1D), and a net expansion of the alveolar macrophage pool was significantly higher than that observed in PI3Kγ KO mice at later time points (120 h post-treatment) (Figure 1C). Of note, control experiments revealed that instillation of vehicle (sterile phosphate-buffered saline/0.1% human serum albumin) into the lungs of mice did not elicit the alveolar accumulation of neutrophils or exudate macrophages into the bronchoalveolar space (24–72 h), thus indicating that neither vehicle *per se* nor the surgical procedure was responsible for the observed effects (data not shown in detail).

To elucidate the role of PI3Kγ activity in circulating monocytes as opposed to sessile lung cells on alveolar exudate macrophage recruitment, chimeric wild-type mice, generated as described in the online supplement, were challenged with PLY (16, 21). Notably, chimeric wild-type mice lacking PI3Kγ activity in circulating leukocytes but not resident lung cells did not recruit

exudate macrophages into the alveolar airspace after challenge with intratracheal PLY (Figure 1E), whereas alveolar neutrophil recruitment was not impaired in these mice (data not shown). In contrast, wild-type mice with a fully intact PI3Kγ system demonstrated a well-coordinated influx of exudate macrophages into the alveolar compartment in response to PLY (Figure 1E). These data demonstrate that inflammatory macrophage recruitment into the alveolar compartment of lungs challenged with PLY alone is critically dependent on PI3Kγ activity in the circulating monocytes.

Effect of PI3Kγ Deletion on Alveolar Exudate Macrophage Recruitment in Response to Intratracheal CCL2

Because PI3Kγ acts downstream of G protein–coupled chemokine receptors, we hypothesized that deletion of PI3Kγ would render circulating monocytes unresponsive to CCL2, the primary monocyte chemoattractant binding to and signaling via CCR2. Unlike wild-type mice, PI3Kγ KO mice failed to elicit monocyte/macrophage recruitment into their lungs upon intratracheal application of CCL2. These data conclusively demonstrate that PI3Kγ is required for CCL2-dependent inflammatory mononuclear phagocyte trafficking *in vivo* (Figure 2A) (16, 23).

To further delineate the role of PI3Kγ in CCL2-dependent monocyte trafficking *in vitro*, BMDMs from wild-type mice or PI3Kγ KO mice were allowed to transmigrate toward optimal concentrations of recombinant CCL2 in the presence of either LY294002, a pan-PI3K inhibitor exerting most of its activity on PI3Kα with no selectivity for any class I PI3K, or a PI3Kγ-specific inhibitor, AS-605240. As shown in Figure 2B, BMDMs exhibited a strong chemotactic response to CCL2 that was only weakly blocked by pretreatment with LY294002, whereas AS-605240 pretreated BMDMs (10 μM) showed markedly reduced chemotaxis to CCL2, and similar results were obtained with correspondingly pretreated, purified peripheral blood monocytes (data not shown). Collectively, these findings underline the importance of PI3Kγ activity in CCL2-dependent monocyte chemotaxis *in vitro* and *in vivo*.

Effect of PI3Kγ Deficiency on the Lung Inflammatory Response to *S. pneumoniae* Infection

Histopathologic examination of lung sections from *S. pneumoniae*–infected PI3Kγ KO and wild-type mice demonstrated a similar degree of developing neutrophilic alveolitis by Day 2 postinfection (Figure 3A). Up until Day 4 of infection, PI3Kγ KO mice developed progressive histopathologic manifestations similar to wild-type mice, as reflected by a strong neutrophilic alveolitis and alveolar fibrinous exudate formation within the lungs, with overall inflamed lung tissue ranging between 40 and 50%. However, marked differences were observed at Day 7 postinfection, at which time virtually all PI3Kγ KO mice demonstrated severe pneumococcal pneumonia affecting whole lung lobes (50–70% inflamed lung tissue), characterized by established purulent pleuritis, fibrinous exudate formation, and neutrophilic necrosis (*see arrows* in Figure 3A, *left*). At this time point, most of the wild-type mice demonstrated resolving pulmonary inflammation with only occasional, focally restricted fibrinous exudates and strongly increased numbers of alveoli-accumulating mononuclear phagocytes, with an overall inflamed lung tissue ranging between 5 and 10% (*see arrows* in Figure 3A, *right*). Notably, severe pneumococcal pneumonia developing in the lungs of PI3Kγ KO mice was also evident from the macroscopic appearance of infected lungs as compared with wild-type control lungs (Figure 3B). Moreover, PI3Kγ KO mice demonstrated an approximately 20% loss of body weight at Day 2 of pneumococcal infection compared with wild-type mice, which only showed a body weight loss of approximately 10% (Figure 3C); wild-type mice largely regained their body weight during the 7-day observation period, whereas *S. pneumoniae*–infected PI3Kγ KO mice were unable to recover from loss of body weight within this period, again reflecting the severe disease progression in these mice.

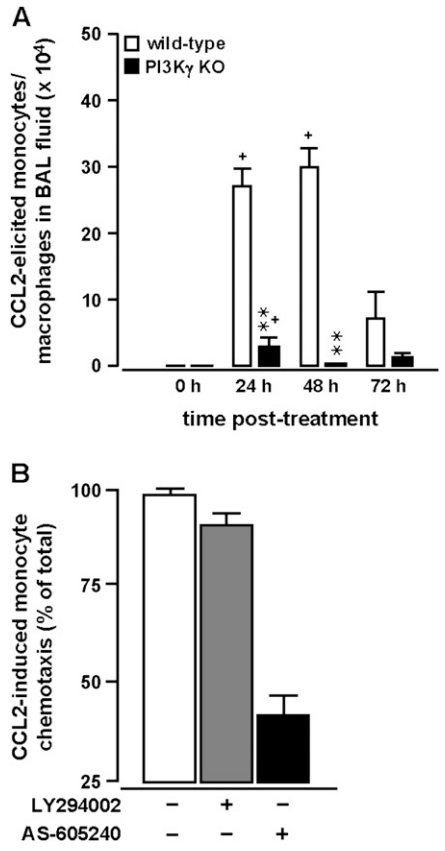

Figure 2. Effect of intratracheal CCL2 application on alveolar monocyte/macrophage trafficking in wild-type versus PI3Kγ knockout (KO) mice. (*A*) Wild-type mice (*open bars*) and PI3Kγ KO mice (*solid bars*) either received vehicle (0 h, phosphate-buffered saline/0.1% human serum albumin) or an intratracheal application of recombinant murine monocyte chemoattractant CCL2 (50 μg/mouse). At the indicated time points, mice were killed and numbers of alveolar recruited monocytes/macrophages were quantified in bronchoalveolar lavage (BAL) fluid. Values are shown as mean ± SD of eight (0 h) or nine mice (24 h) or five mice (48 h, 72 h) per group. $^{+}$Significant increase ($p < 0.05$) compared with the respective 0 h control values; *double stacked asterisks*, significant difference ($p < 0.01$) between PI3Kγ KO and wild-type mice. (*B*) Bone marrow–derived monocytes collected from untreated wild-type mice were starved in serum-free medium for 3 hours, and then allowed to transmigrate toward optimal CCL2 gradients (1 nM) in the absence (*open bar*) or presence of either the pan-PI3K inhibitor LY 294002 (*gray bar*) or the PI3Kγ-specific inhibitor AS-605240 (10 μM, *solid bar*) for 3 hours. Subsequently, the transmigrated monocytes were collected from the lower compartment of transwell chambers and quantified with a Beckman Coulter AcT 5diff (Beckman Coulter, Krefeld, Germany). Values are shown as mean ± SD of three independent experiments.

Effect of Deletion or Pharmacologic Inhibition of PI3Kγ on Lung Neutrophil and Mononuclear Phagocyte Recruitment and Bacterial Clearance Capacity in Pneumococcal Pneumonia

We next determined the lung pneumococcal clearance capacity in PI3Kγ KO and wild-type mice either in the absence or presence of the PI3Kγ-specific inhibitor AS-605240. Both PI3Kγ

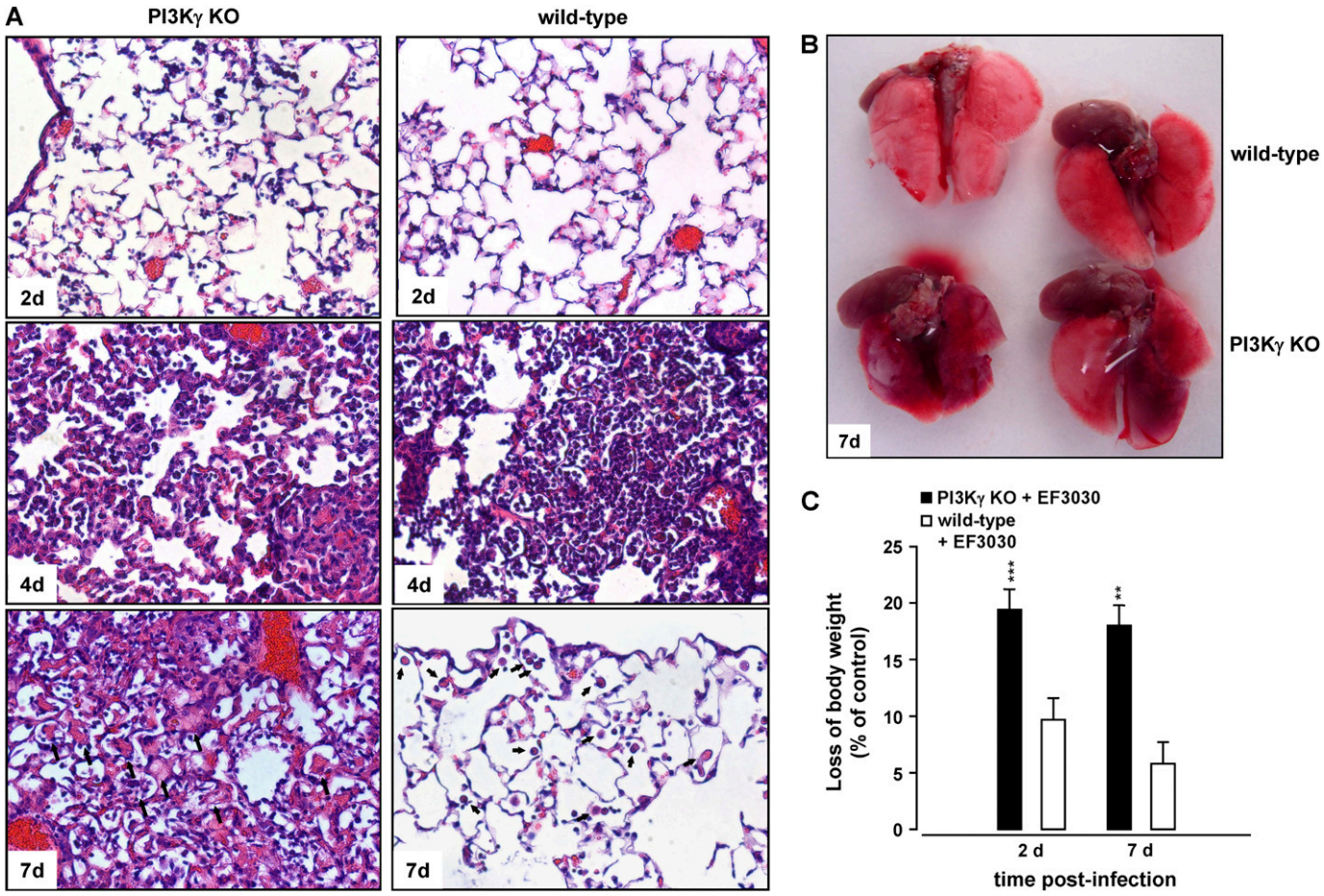

Figure 3. Lung histopathology of PI3Kγ knockout (KO) and wild-type mice infected with *S. pneumoniae*. (*A*) PI3Kγ KO and wild-type mice were infected with *S. pneumoniae* (EF3030; $\sim 4 \times 10^7$ cfu/mouse), as indicated. At the given time points, mice were killed and the lungs carefully removed and subjected to histopathologic examination. Lung sections (5 μm) were stained with hematoxylin–eosin. Representative lung histopathologies from *S. pneumoniae*–infected PI3Kγ KO (*left panels*) and wild-type mice (*right panels*) are shown at an original magnification of ×20. *Arrows* indicate either fibrinous exudate formations (7 d, PI3Kγ KO, *left*) or accumulations of alveolar macrophages (7 d, wild-type, *right*). (*B*) Lungs were removed from *S. pneumoniae* infected PI3Kγ KO and wild-type mice at Day 7 post-treatment and submersed in phosphate-buffered saline for photographic illustration. Photographs were taken with a digital camera (Nikon, Kawasaki, Japan) operating at a 5-megapixel resolution. (*C*) Changes in body weights of PI3Kγ KO (*solid bars*) and wild-type mice (*open bars*) infected with *S. pneumoniae* (EF3030; $\sim 4 \times 10^7$ cfu/mouse). The data represent mean values of five independent determinations per group and time point. *Double stacked asterisks*, $p < 0.04$; *triple stacked asterisks*, $p < 0.02$ versus wild-type controls.

KO and wild-type mice pretreated with AS-605240 compound demonstrated a strongly reduced lung pneumococcal clearance capacity compared with wild-type mice, resulting in greater than 10-fold higher bacterial loads in AS-605240–pretreated, *S. pneumoniae*–infected mice as compared with control mice by 48 hours postinfection (Figures 4A and 4B). Importantly, this reduced pneumococcal clearance was not attributable to reduced alveolar neutrophil recruitment either in null or PI3Kγ inhibitor–pretreated wild-type mice (Figures 4C and 4D).

Effect of PI3Kγ Deficiency on Alveolar Exudate Macrophage Recruitment and Bacterial Clearance Capacity in Pneumococcal Pneumonia

We also determined the role of PI3Kγ in alveolar exudate macrophage recruitment in response to *S. pneumoniae* infection. Exudate macrophages were distinguished from resident alveolar macrophages using their differential β2 integrin CD11b and CD11c cell surface antigen expression profile (Figure 5A). Gating of BAL fluid macrophages according to their forward scatter (FSC) versus side scatter (SSC) and FSC versus F4/80 antigen expression (Figure 5A, *left* and *right dot plots*) allowed us to identify resident alveolar macrophages from untreated and *S. pneumoniae*–infected (96 h) mice (P1), according to their $CD11c^+/CD11b^-$ cell surface antigen expression (P1 in Figure 5A, *left* and *right dot plots*), whereas monocyte-derived exudate macrophages were found to be CD11c positive and strongly CD11b positive (P2 in Figure 5A, *right dot plot*), thereby allowing their flow cytometric differentiation from resident alveolar macrophages (P1 in Figure 5A, *right dot plot*). Both resident alveolar and exudate macrophages lacked major histocompatibility class II and CD86 antigen expression, thus confirming their macrophage phenotype (histograms P1 [*left side*] and P1 and P2 [*right side*] of Figure 5A).

Both PI3Kγ KO mice and wild-type mice pretreated with the PI3Kγ-specific inhibitor AS-605240 demonstrated significantly impaired alveolar macrophage exudate recruitment in response to pneumococcal infection (Figures 5B and 5C), which was accompanied by a strong depletion of resident alveolar

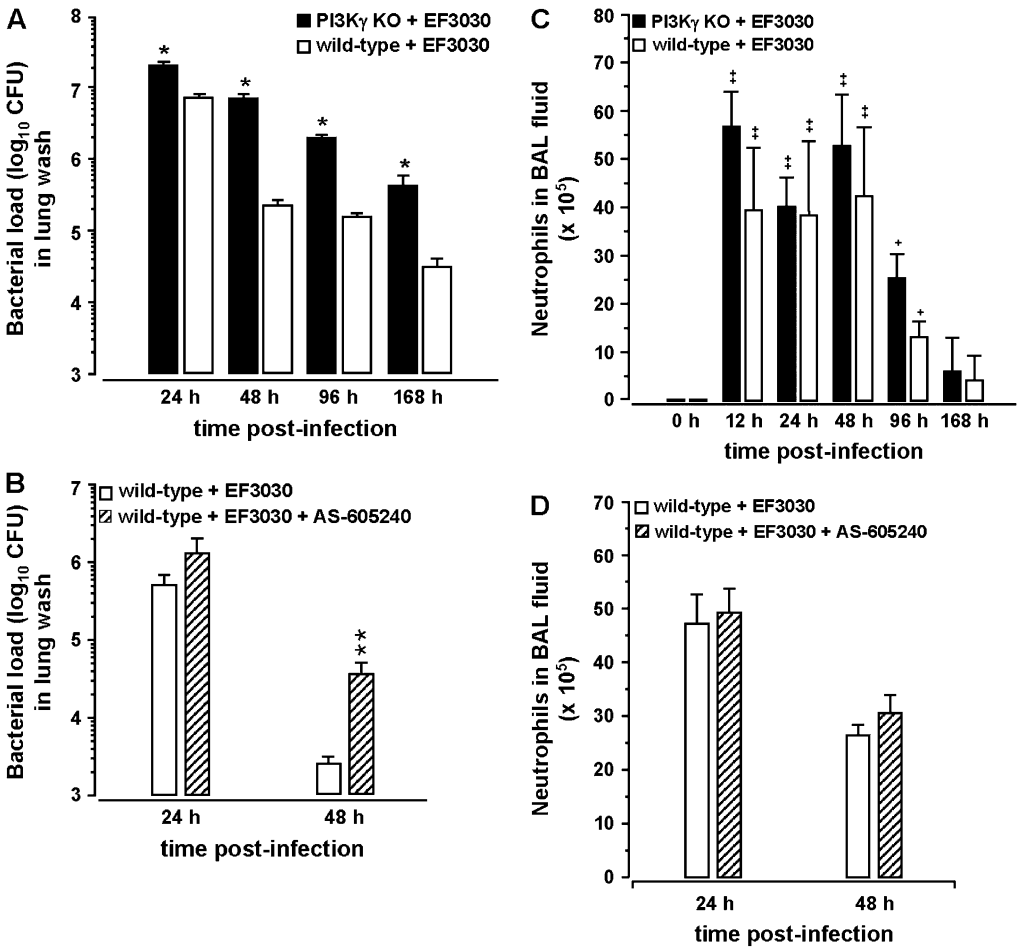

Figure 4. Pneumococcal clearance capacities and alveolar neutrophil recruitment profiles in PI3Kγ knockout (KO) and wild-type mice in the absence or presence of AS-605240. PI3Kγ KO mice (*solid bars*) or wild-type mice (*open bars*) were infected with *S. pneumoniae* (EF3030) (*A*) via intratracheal application, or wild-type mice were infected with *S. pneumoniae* (EF3030) via intratracheal application either in the absence (*open bars*) or presence (*hatched bars*) of the PI3Kγ-specific inhibitor AS-605240 (*B*) for the indicated time points. Subsequently, mice were killed and bacterial counts were determined from whole-lung washes (*A, B*), or bronchoalveolar lavage (BAL) fluid neutrophil counts were calculated from leukocyte differentials using Pappenheim-stained cytospin preparations (*C, D*), as indicated. The data represent the mean ± SD of nine (24 h, 48 h) or five mice (96 h, 168 h) (*A, C*) or 10 mice (*B, D*) per group and time point. *Single* and *double stacked plus signs* indicate $p < 0.05$ and $p < 0.01$, compared with the respective 0 h control values; *single* and *double stacked asterisks* indicate $p < 0.05$ and $p < 0.04$, respectively, versus wild-type control.

macrophages at 12 hours up to 48 hours postinfection (Figure 5C), thus strongly supporting the concept that PI3Kγ activity is an essential component of lung mononuclear phagocyte trafficking during pneumococcal pneumonia. To further assess the role of elicited exudate macrophages in the early onset of pneumococcal pathogen elimination, wild-type mice were infected with *S. pneumoniae* either in the absence or presence of the function-blocking anti-CCR2 antibody MC21. Interestingly, although MC21 application nearly completely inhibited the inflammatory exudate macrophage influx into the alveolar airspace of infected mice, bacterial loads determined in lung washes of infected wild-type mice did not show any significant changes compared with control mice (Figures 6A and 6B).

Survival of PI3Kγ KO versus Wild-Type Mice Subsequent to *S. pneumoniae* Infection

Finally, we analyzed the effect of PI3Kγ deletion on the survival of mice infected with *S. pneumoniae* D39. Importantly, we found that wild-type mice demonstrated a significantly prolonged survival upon infection with 2×10^3 cfu D39 as compared with PI3Kγ KO mice within the 7-day observation period (Figure 7).

DISCUSSION

Using both gene KO and pharmacologic approaches, we investigated the role of PI3Kγ in lung inflammation developing in response to both PLY (one of the most important pneumococcal virulence factors) and *S. pneumoniae* infection in mice. PI3Kγ KO and wild-type mice pretreated with the PI3Kγ-specific inhibitor AS-605240 responded with a neutrophilic alveolitis, but showed an impaired exudate macrophage recruitment in response to PLY and *S. pneumoniae* infection. Both null and AS-605240–inhibitor pretreated wild-type mice displayed a strongly reduced pneumococcal clearance and showed a reduced resolution/repair process, most probably due to defects in mounting an appropriate mononuclear phagocyte recruitment to *S. pneumoniae* challenge. Collectively, the data show that either deletion or blockade of PI3Kγ impairs central innate host defense mechanisms of the lung upon challenge with the prototypic gram-positive bacterial pathogen *S. pneumoniae*.

Alveolar mononuclear phagocyte recruitment in response to intratracheal PLY challenge was recently found to critically depend on the CCL2–CCR2 axis (14), and for the first time, the data presented in the current study specify a role of PI3Kγ in the PLY-triggered CCL2–CCR2 downstream signaling cascade evoked in monocytes *in vivo*. This is based on the findings that PI3Kγ null exudate macrophages were unable to accumulate within the alveolar airspace of CCL2-treated mice, just as they were unable to accumulate within the alveolar airspace of chimeric wild-type mice challenged with PLY, which again highlights the particular importance of PI3Kγ activity in circulating leukocytes (2, 8, 24) but not sessile endothelial cells (25) for the PLY-induced monocyte extravasation process *in vivo*. In addition, for the first time, we have shown that both BMDMs and highly purified primary blood monocytes did not migrate toward CCL2 chemokine gradients in the presence of the PI3Kγ-specific inhibitor AS-605240 *in vitro*, whereas inhibition of P13K by LY294002 did not affect monocyte chemotaxis, thus further highlighting

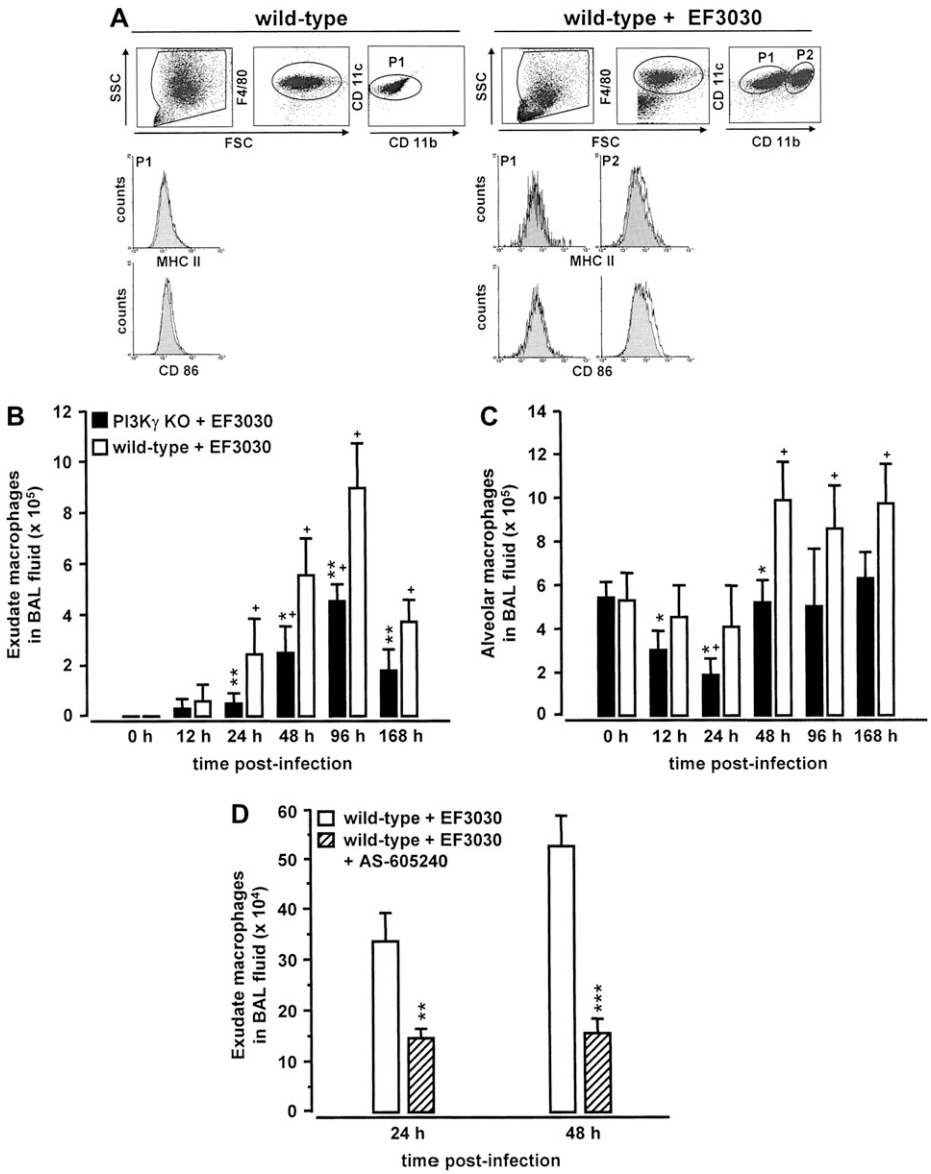

Figure 5. Effect of deletion or pharmacologic blockade of PI3Kγ on the alveolar mononuclear phagocyte recruitment in response to *S. pneumoniae* infection. (*A*) Flow cytometric discrimination of resident alveolar macrophages (P1) and exudate macrophages (P2) in bronchoalveolar lavage (BAL) fluids of untreated wild-type mice (*left dot plots* and *histograms*) or wild-type mice infected with *S. pneumoniae* (EF3030) for 4 days (*right dot plots* and *histograms*). Macrophages were gated according to their FSC/SSC (forward scatter/side scatter) and FSC/F4/80 characteristics, as indicated. Exudate macrophages (P2) were distinguished from resident alveolar macrophages (P1) according to their CD11b and CD11c antigen expression profile (P2, *right dot plot*), while resident alveolar macrophages were CD11c but not CD11b positive (P1 in *left* and *right dot plots*). (*B*) PI3Kγ knockout (KO) mice (*solid bars*) and wild-type mice (*open bars*) were infected with *S. pneumoniae* (EF3030) via intratracheal application. At the indicated time points, mice were killed and subjected to BAL for determination of inflammatory recruited exudate macrophages (*B*) and resident alveolar macrophages (*C*). In (*D*), vehicle-treated wild-type mice or wild-type mice pretreated with the PI3Kγ-specific inhibitor AS-605240 were infected with *S. pneumoniae* (EF3030) via intratracheal instillation. At 24 and 48 h postinfection, mice were killed and subjected to BAL for determination of inflammatory recruited exudate macrophages. The data are shown as mean ± SD of 5 (*A*–*C*) or 10 mice (*D*) per group and time point. $^{+}$Significant increase/decrease ($p < 0.05$) compared with the respective 0 h control values. *Single*, *double stacked*, and *triple stacked asterisks* indicate $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, versus wild-type controls.

the particular importance of the γ isoform of PI3K in the CCL2-induced downstream signaling cascade.

PLY challenge of wild-type mice and PI3Kγ KO mice induced a strong depletion of resident alveolar macrophages. This PLY effect on alveolar macrophages was recently found to be attributable to its pore-forming, cytotoxic activities, because its noncytotoxic derivative, PdB (PLY with a Trp_{433}-Phe mutation), lacking the pore-forming activity of PLY, was not able to deplete macrophages *in vivo* (14, 18). PI3Kγ KO as opposed to wild-type mice also demonstrated a significantly delayed repopulation of the depleted alveolar macrophage pool, and although our experiments with anti-CCR2 antibodies to block inflammatory monocyte recruitment did not support a direct contribution of recruited monocytes/macrophages to the early bacterial clearance, such a perturbation of alveolar macrophage homeostasis may at least indirectly promote the bacterial spread within distal airspaces by attenuating resolution/repair processes after pneumococcal infection. In line with this, previous publications have shown that liposomal clodronate–mediated alveolar macrophage depletion significantly affected survival rates in various bacterial lung infection models in mice (26–28). Very similar to the effects seen with the purified pathogen-associated molecular pattern PLY, infection of the mice with PLY-producing *S. pneumoniae* also caused a transient depletion of alveolar macrophages in both PI3Kγ KO and wild-type mice pretreated with AS-605240. Macrophages from PI3Kγ KO or inhibitor-pretreated wild-type mice appear not to be more sensitive to depletion by *S. pneumoniae* than wild-type macrophages, because *S. pneumoniae*–infected wild-type mice pretreated with the anti-CCR2 antibody MC 21 also responded with a strongly depleted alveolar macrophage pool upon pneumococcal challenge (unpublished observations). Interestingly, this transient drop in alveolar macrophages noted in the current study had no effect on the neutrophilic alveolitis that developed in the two experimental groups. Despite this, both null and inhibitor-pretreated wild-type mice had a nearly 10-fold lower pneumococcal clearance in their lungs compared with control animals, most probably due to killing defects in the recruited PI3Kγ-null neutrophils (2), which is supported by our recent observations in which PI3Kγ-deficient neutrophils were found to be defective in respiratory burst and reactive oxygen species production triggered by bacterial peptides *in vitro* (2). Such defective respiratory burst machinery also very likely

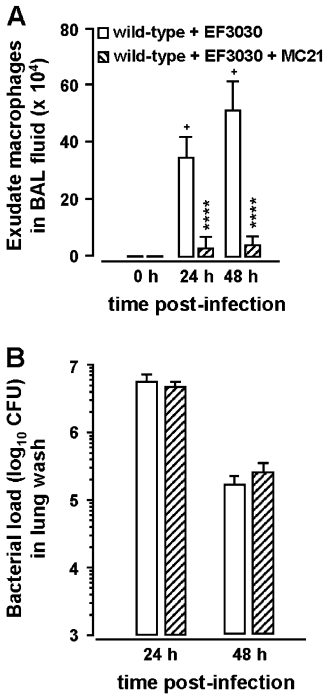

Figure 6. Effect of CCR2 inhibition on alveolar exudate macrophage recruitment and lung pneumococcal clearance in wild-type mice. (*A*) For CCR2 inhibition studies, mice received intraperitoneal injections of the function-blocking anti-CCR2 antibody MC21 3 hours before and every 24 hours subsequent to intratracheal infection with *S. pneumoniae* EF3030 for the indicated time points. Subsequently, mice were subjected to bronchoalveolar lavage (BAL) for quantification of alveolar recruited exudate macrophages (*A*) and bacterial loads in lung washes (*B*), respectively. The data are shown as mean ± SD of five mice per group and time point. $^{+}$Significant increase ($p < 0.05$) compared with the respective 0 h control values. *Single*, *double stacked*, and *triple stacked asterisks* indicate $p < 0.05$, $p < 0.02$, and $p < 0.003$, respectively, versus wild-type controls.

underlies the defective pneumococcal killing observed in the current study *in vivo*.

In contrast to the strongly reduced exudate macrophage recruitment, deletion or pharmacologic blockade of PI3Kγ did not affect the development of neutrophilic alveolitis, both in PLY-challenged or *S. pneumoniae*–infected mice. Another study also demonstrated lack of suppressed lung tissue sequestration of PI3Kγ-deficient neutrophils compared with wild-type neutrophils upon peritoneal *E. coli* sepsis, possibly due to increased CD47 (integrin-associated protein) and β3 integrin association of PI3Kγ-null neutrophils with extracellular vitronectin (29). On the other hand, lack of PI3Kγ activity was found to impair neutrophil trafficking to LPS-inflamed mouse lungs (25). Thus, the different inflammatory stimuli and experimental models used (acute vs. chronic; *E. coli* vs. *S. pneumoniae*) may well explain the differential contribution of PI3Kγ in inflammatory neutrophil trafficking *in vivo*. In addition, it is conceivable that alternative PI3K signaling pathways such as that mediated by PI3Kδ may explain the observed lack of effect of PI3Kγ inhibition on the neutrophil recruitment process to *S. pneumoniae*–induced lung infection.

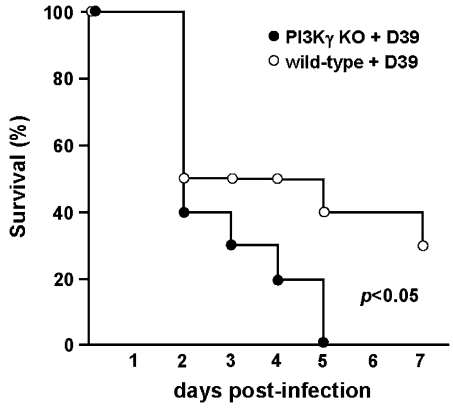

Figure 7. Survival of PI3Kγ knockout (KO) and wild-type mice subsequent to *S. pneumoniae* lung infection. PI3Kγ KO and wild-type mice (n = 10 each) were infected intratracheally with *S. pneumoniae* serotype 2 strain D39 (2×10^3 cfu/mouse), and survival was monitored two times daily during the 7-day observation period. $p < 0.05$ increased survival in wild-type compared with PI3Kγ KO mice.

Histopathologic examinations of lung tissue sections from *S. pneumoniae*–infected PI3Kγ KO mice showed a substantial degree of lung neutrophil necrosis within distal airspaces. Resident lung mononuclear phagocytes are central to the resolution/repair phase in bacterial lung infection, including the elimination of necrotic material from distal airspaces (28, 30). Perturbations of the mononuclear phagocyte system observed in the current study in *S. pneumoniae*–infected PI3Kγ KO mice and wild-type mice pretreated with AS-605240 most probably contributed to the inefficient resolution/repair phase subsequent to pneumococcal infection, ultimately leading to progressive pneumonia. This concept may be supported by two observations. First, in a recent study, liposomal clodronate application to deplete alveolar macrophages was reported to elicit an aggravated lung inflammatory response to *S. pneumoniae* infection, partially due to the reduced resolution/repair mechanisms in macrophage-depleted *S. pneumoniae*–infected lungs (28, 31). Second, ongoing experiments in *S. pneumoniae*–infected wild-type mice pretreated with anti-CCR2 antibody MC21 for 7 days revealed a strongly reduced inflammatory mononuclear phagocyte mobilization and a heavily attenuated resolution/repair phase, ultimately resulting in progressive lobar pneumonia (32). In this context, experiments with PI3Kγ KO mice infected with the serotype 2 pneumococcal strain D39, which is known to progress into septic pneumococcal disease, illustrates the possible consequences for the infected host.

Targeting PI3Kγ signaling is a promising approach in the treatment of chronic inflammatory diseases. However, in view of a clinical application of small-molecule PI3Kγ inhibitors, target validation will be an important future aspect to discriminate between specific effects of the drug and potential side effects (33). Although intracellular signaling pathways are considered to be redundant, the current report shows that alveolar macrophage homeostasis subsequent to pneumococcal challenge is significantly reduced in both null and inhibitor-pretreated mice, supporting the view that PI3Kγ signaling represents a central component in inflammatory mononuclear phagocyte mobilization after pneumococcal infection.

In summary, in view of a clinical application of PI3Kγ inhibitors for the treatment of chronically ill patients, the current data point to potentially evolving side effects of PI3Kγ inhibition that may render the lung innate host defense system less effective to adequately respond to lower respiratory tract infections.

Conflict of Interest Statement: U.A.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. C.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.K.S. is employed by Merck Serono. T.R. is employed by Merck Serono. J.C.P. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. D.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. T.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. R.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. R.M.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. W.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. C.R. is

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Anlage 2

Winter, C., K. Taut, M. Srivastava, F. Länger, M. Mack, D. E. Briles, J. C. Paton, R. Maus, T. Welte, M. D. Gunn, U. A. Maus

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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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[1]

Christine Winter,* Katharina Taut,* Mrigank Srivastava,* Florian Länger,† Matthias Mack,‡ David E. Briles,§ James C. Paton,¶ Regina Maus,* Tobias Welte,* Michael D. Gunn,‖ and Ulrich A. Maus[2]*

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.**

The Gram-positive *Streptococcus pneumoniae* is a commensal bacterium colonizing the nasopharynx of ~30–50% of the healthy human population. *S. pneumoniae* is also the leading causative pathogen in community-acquired pneumonia that frequently progresses to invasive pneumococcal disease and is associated with high morbidity and mortality rates worldwide. Infections of the lung with *S. pneumoniae* are characterized by a rapidly mounted neutrophilic alveolitis, as a critical component of the antimicrobial host defense to prevent pneumococcal spread within distal airspaces (1). In addition, resident and recruited mononuclear phagocytes are considered to play critical roles in resolution/repair processes during bacterial infections by phagocytosing invading bacteria, apoptotic/necrotic neutrophils, and cellular debris to re-establish organ homeostasis (1, 2). Recent reports from our group demonstrated that intratracheal application of the pneumococcal cytolysin pneumolysin not only caused severe lung edema, but also transiently depleted the resident alveolar macrophage pool in mice. This drop in alveolar macrophage numbers noted in pneumolysin-challenged mice was followed by a CCR2-dependent de novo recruitment of mononuclear phagocytes into the alveolar compartment. Such mononuclear phagocyte mobilization could be blocked either by application of function-blocking anti-CCR2 Ab MC21 (3) or by application of orally available pharmacological inhibitors of PI3Kγ acting as an integral component of the CCR2 downstream signaling cascade (34). These data gave rise to the concept that interfering with signaling pathways that regulate inflammatory mononuclear phagocyte recruitment would impair the resolution/repair processes normally developing in response to inhaled bacterial pathogens, while experimentally increasing mononuclear phagocyte trafficking toward *S. pneumoniae*-infected lungs might improve the innate host defense capacity of the lung to pneumococcal infections.

In the current study, we hypothesized that increased CCL2-dependent mononuclear phagocyte recruitment toward the lungs of mice would improve the resolution/repair process in response to *S. pneumoniae* infection. To test this hypothesis, wild-type mice and transgenic mice exhibiting constitutively increased mononuclear phagocyte immigration into the alveolar air space due to lung-specific overexpression of human CCL2 in type II alveolar epithelial cells were infected with *S. pneumoniae* in the absence or presence of anti-CCR2 Abs. The resultant inflammatory response to pneumococcal challenge was analyzed.

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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% CO_2 for 18 h and subsequent determination of CFU.

Infection of CCL2 overexpressing and wild-type mice with *S. pneumoniae* was done using freshly prepared dilutions of thawed aliquots adjusted to $\sim 3 \times 10^7$ 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 $\sim 2 \times 10^7$ 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).[3] 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% CO_2 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.

[3] Abbreviations used in this paper: BAL, bronchoalveolar lavage; ARDS, acute respiratory distress syndrome; DC, dendritic cell; MHC-II, MHC class II; FSC, forward light scatter; SSC, side light scatter.

BAL and preparation of lung parenchymal tissue

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 DNase I 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 with specificities for the following cell surface molecules (9): PerCP Cy5.5-conjugated anti-CD11b, PE Cy5.5- or allophycocyanin-conjugated anti-CD11c, PE-conjugated anti-CD49d, 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-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. Lung tissue samples were paraffin-embedded, and lung sections of 5 μm were stained with H&E and Elastica-van-Gieson and examined histopathologically using a Zeiss Axiovert 200 M microscope.

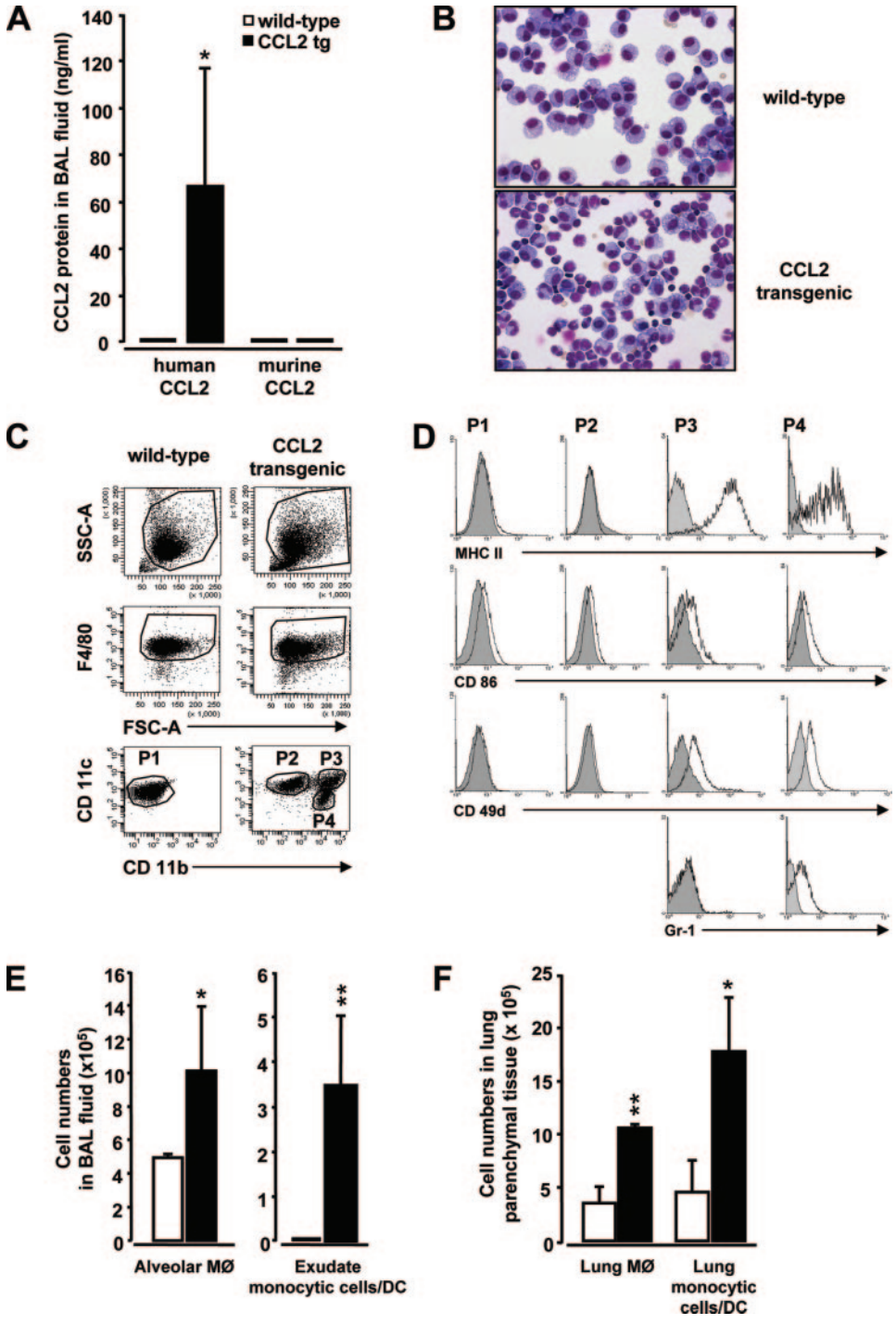

FIGURE 1. Effect of CCL2 overexpression on lung mononuclear phagocyte subset accumulations. *A*, Analysis of human and murine CCL2 protein levels in BAL fluids of untreated CCL2 overexpressing and wild-type mice. *B*, Pappenheim-stained cytospin preparations of BAL fluid cellular constituents of untreated 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 $CD11b^{neg}$, $CD11c^{pos}$ 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 $MHC\text{-}II^{neg}$, $CD86^{low}$, $CD49d^{neg}$ phenotype, whereas cells in P3 and P4 subsets were characterized as monocyte-derived myeloid DC ($MHC\text{-}II^{high}$, $CD86^{pos}$, $CD49d^{pos}$, $Gr\text{-}1^{neg}$) and alveolar monocytic cells ($MHC\text{-}II^{neg\text{-}low}$, $CD86^{low}$, $CD49d^{pos}$, $Gr\text{-}1^{pos}$), 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.

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. 1*A*,

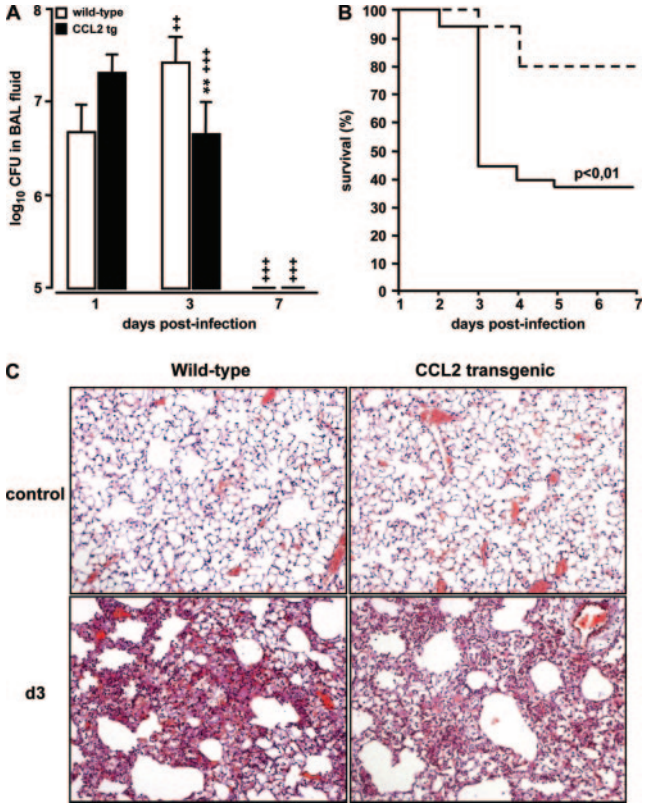

FIGURE 2. Bacterial clearance and survival in CCL2 overexpressing mice and wild-type mice infected with *S. pneumoniae*. Wild-type mice (□) and CCL2 overexpressing mice (■) were infected with ~3 × 10^7 CFU *S. pneumoniae*. At the indicated time points, bacterial loads in whole lung washes (*A*) or the percentage of survival (*B*) in wild-type (solid line) (n = 20 mice/group) or CCL2 overexpressing (dashed line) mice was determined during an observation period of 7 days. ++, $p < 0.01$; +++, $p < 0.001$ compared with the respective CFU values at day 1 postinfection. **, $p < 0.01$ compared with wild-type mice. *C*, Histopathological examination of lung tissue sections collected from either wild-type mice (*left*) or CCL2 overexpressing mice (*right*) left uninfected (control) or analyzed by day 3 postinfection (d3). Photomicrographs were taken at an original magnification of ×10 using an Axiovert 200 M microscope (Zeiss). Data are the mean ± SD per group (n = 8, CCL2 transgene; n = 13, wild-type) and per time point.

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 cytospins 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 were composed of alveolar macrophages and cells with a monocytic morphology, as well as a minor portion of lymphocytes (Fig. 1*B*) (4). FACS analysis of mononuclear phagocytes contained in BAL fluids of wild-type mice confirmed these cells to be "classical" alveolar macrophages, due to their FSC vs SSC characteristics in conjunction with an F4/80^{pos}, CD11c^{high}, CD11b^{neg}, MHC-II^{neg}, CD86^{low}, and CD49d^{neg} cell surface Ag expression profile (Fig. 1, *C* and *D*, P1), which is consistent with recent reports (10, 11). In contrast, FACS analysis of mononuclear phagocytes contained in BAL fluids of untreated CCL2 overexpressing mice revealed three distinct phagocyte populations depicted in Fig. 1*C* exhibiting a differential CD11c vs CD11b cell adhesion molecule expression profile (Fig. 1*C*, P2–P4). Cells of population P2 were found to be CD11c^{pos} but CD11b^{neg}, MHC-II^{neg}, CD86^{low}, and CD49d^{neg}, thus again representing classical alveolar macrophages also detected in control mice (Fig. 1, *C* and *D*, P2). The second mononuclear phagocyte subset recovered from the lungs of CCL2 overexpressing mice showing a monocytic morphology (Fig. 1*B* and population P3 in Fig. 1*C*) revealed a differential Ag expression profile (CD11c^{high}, CD11b^{high}, MHC-II^{high}, CD86^{pos}, CD49d^{pos}, but Gr-1^{neg}), thus characterizing these cells as monocyte-derived myeloid DC (Fig. 1, *C* and *D*, P3), consistent with recent reports (11, 12). Cells of population P4 were found to be CD11b^{high}, CD11c$^{neg\text{-}int}$, MHC-II$^{neg\text{-}low}$, CD86^{low}, CD49d^{pos}, and Gr-1^{int}, thus representing alveolar monocytic cells (Fig. 1, *C* and *D*). Based on this immunophenotypic characterization of mononuclear phagocyte subsets identified in the two treatment groups, we found that CCL2 overexpressing mice exhibited an ~2-fold expanded resident alveolar macrophage pool and a strongly expanded pool of exudate alveolar monocytic cells/DC in their bronchoalveolar space compared with wild-type mice (Fig. 1*E*). 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. 1*F*.

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. 2*A*). Importantly, this improved pneumococcal clearance observed in CCL2 overexpressing mice was associated with a significantly increased survival. As shown in Fig. 2*B*, ~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. 2*C*).

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 chemoattractants 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

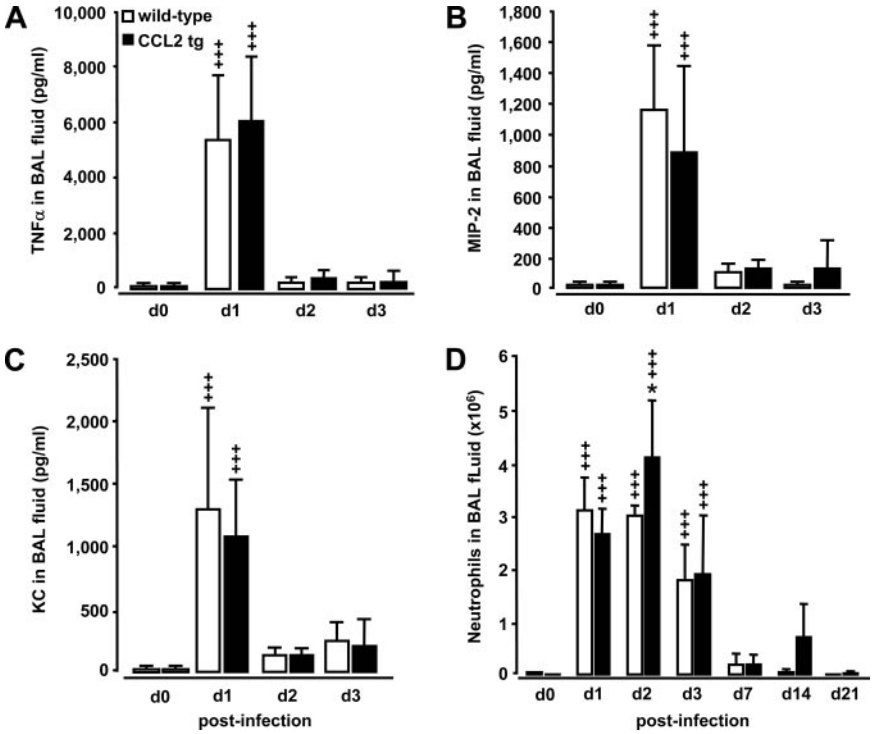

FIGURE 3. Proinflammatory cytokine responses and neutrophilic alveolitis in CCL2 overexpressing compared with wild-type mice infected with *S. pneumoniae*. Wild-type mice (□) and CCL2 overexpressing mice (■) were infected with *S. pneumoniae* for various time points. Subsequently, mice were subjected 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.

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. 3*D*). 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.

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. 4*A*). 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. 4*B*). 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^{pos}, CD11c^{high}, CD11b^{neg}, MHC-II^{neg}, CD86^{neg}, CD49d^{neg} cell surface Ag expression profile, consistent with the mononuclear phagocytes detected under baseline conditions

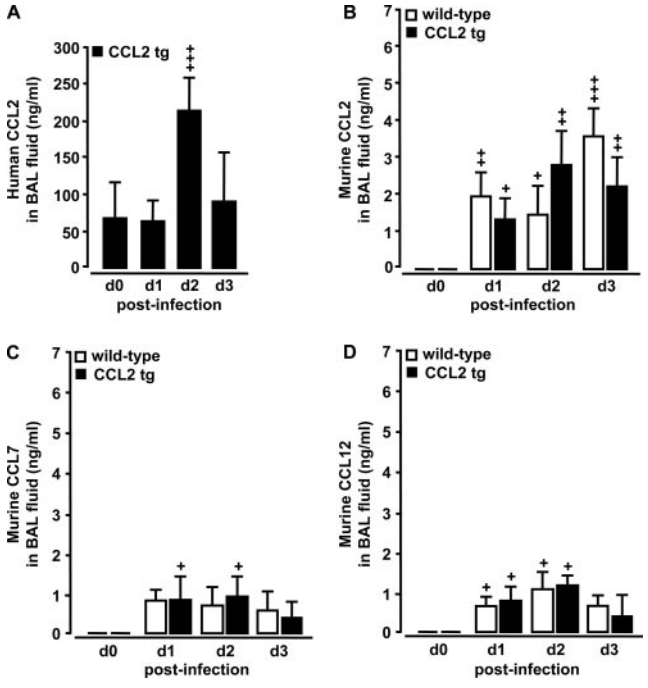

FIGURE 4. Release of monocyte chemoattractants in CCL2 overexpressing and wild-type mice infected with *S. pneumoniae*. CCL2 overexpressing mice and wild-type mice were either uninfected (d0) or were infected with *S. pneumoniae* for various time points. Subsequently, mice were subjected to BAL and determination 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.

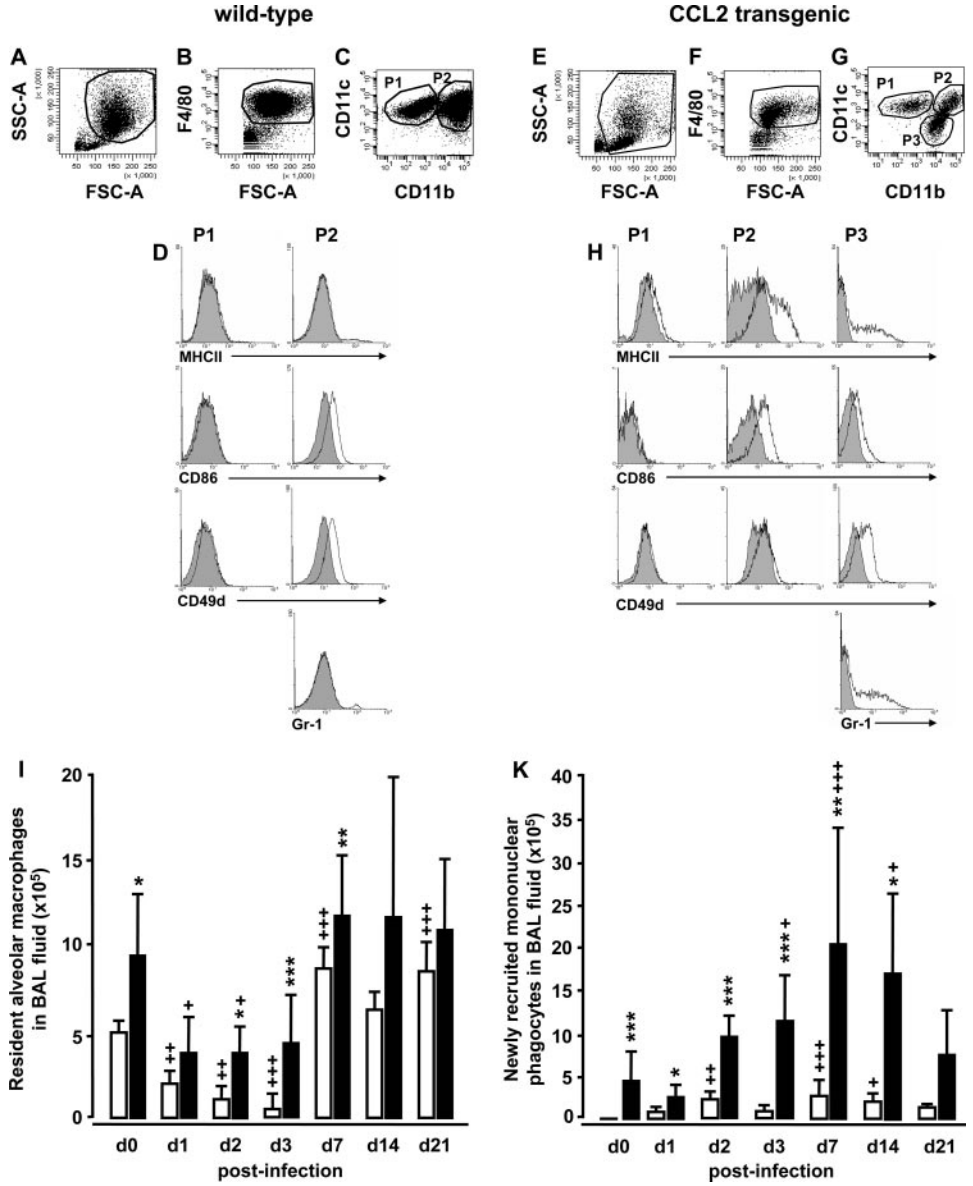

FIGURE 5. Mononuclear phagocyte subset recruitment in CCL2 overexpressing and wild-type mice challenged with *S. pneumoniae*. Dot plot (*A–C*) and histogram (*D*) analysis of populations (P) of mononuclear phagocyte subsets of alveolar macrophages (P1) and inflammatory elicited macrophages (P2) contained in BAL fluids of wild-type mice at day 7 postinfection. Corresponding dot plot (*E–G*) and histogram (*H*) analyses of population of mononuclear phagocyte subsets alveolar macrophages (P1) and alveolar DC (P2) ($CD11c^{pos}$, $CD11b^{pos}$, MHC-II^{high}) and inflammatory-elicited monocytes (P3) ($CD11c^{neg}$, $CD11b^{pos}$, MHC-$II^{neg\text{-}low}$) recovered from the alveolar compartment of *S. pneumoniae*-challenged CCL2 overexpressing mice at day 7 postinfection. Cells shown in *A* and *E* (P1–P3) were gated according to their FSC vs SSC characteristics and in *B* and *F* according to their FSC vs F4/80 expression to exclude contaminating neutrophils from further analysis, followed by a subset-specific gating according to their differential CD11b vs CD11c Ag expression profiles (*C* and *G*). Total cell number of either resident alveolar macrophages (*I*) or recruited mononuclear phagocyte subsets (*K*) recovered from the lungs of untreated or *S. pneumoniae*-challenged wild-type mice (□) or CCL2 overexpressing mice (■) at day 7 postinfection is shown. Note that in *K*, the given values (■) represent the combined total cell number of alveolar DC and inflammatory monocytes (P2 and P3) recovered from *S. pneumoniae*-infected CCL2 overexpressing mice over time. Data shown are mean ± SD of five mice per group and time point. +, $p < 0.05$; ++, $p < 0.01$; +++, $p < 0.001$) compared with the respective control values. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with wild-type mice.

(compare with Fig. 1, *C* and *D*). The second population of exudate mononuclear phagocytes showed a monocytic morphology in Pappenheim-stained cytospins (data not shown) and demonstrated a differential $CD11c^{high}$ and $CD11b^{high}$ Ag expression profile, but were consistently found to be MHC-II^{neg}, in conjunction with $CD86^{low}$, $CD49d^{pos}$, and Gr-1^{neg}, thus representing inflammatory recruited monocyte-derived exudate macrophages (Fig. 5, *C* and *D*, P2). 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*) characterized by their $CD11b^{high}$ but $CD11c^{neg\text{-}low}$, MHC-$II^{neg\text{-}low}$, $CD86^{low}$, $CD49d^{pos}$, and Gr-$1^{neg/pos}$ 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

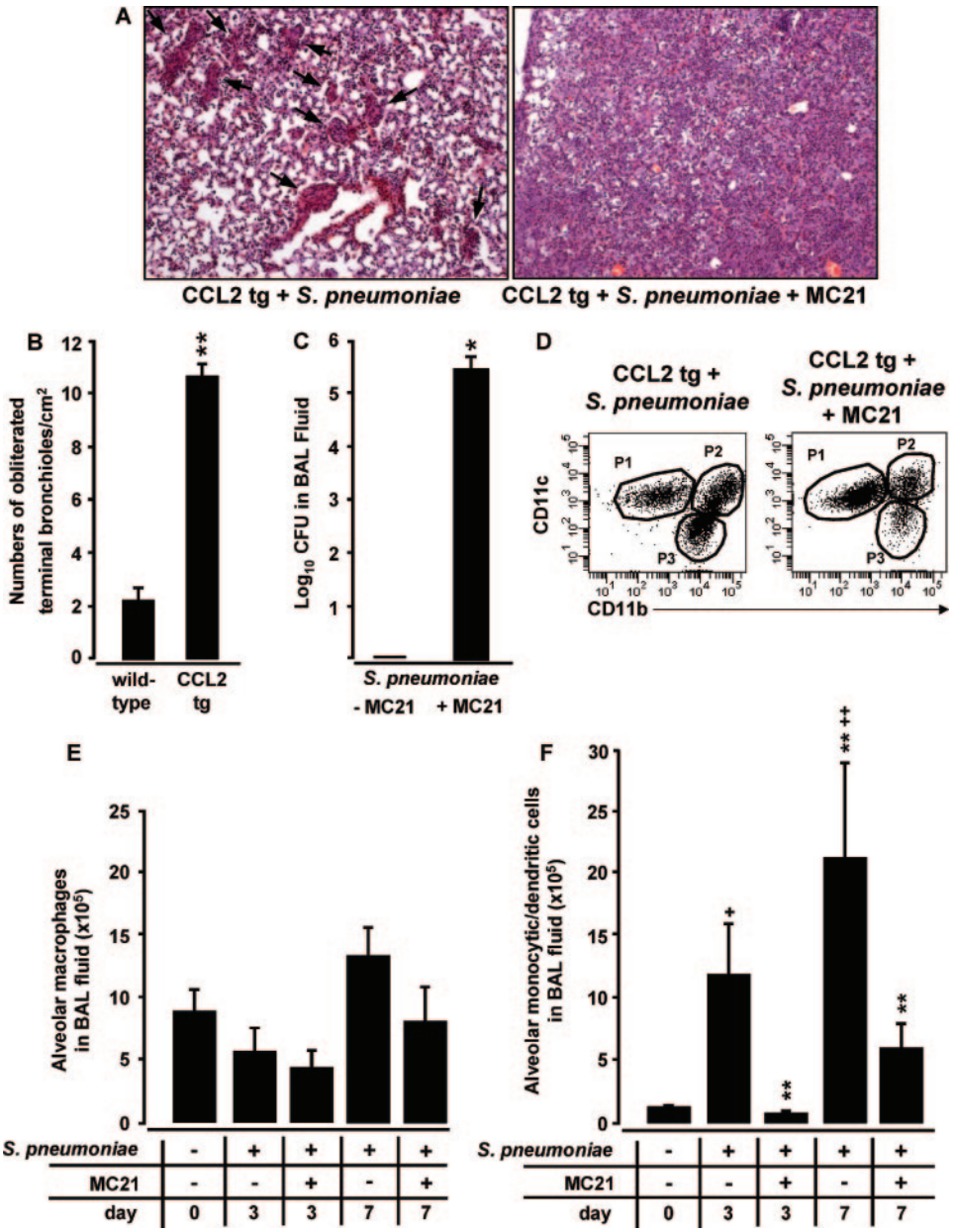

FIGURE 6. Effect of CCR2 blockade on lung histopathology, pneumococcal clearance, and mononuclear phagocyte mobilization in *S. pneumoniae*-infected CCL2 overexpressing mice. *A*, Lung histopathology of CCL2 overexpressing mice infected with *S. pneumoniae* for 7 days either in the absence (*left*) or 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 *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 *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 *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 *S. pneumoniae* only infected CCL2 overexpressing mice. +, $p < 0.05$; ++, $p < 0.01$ compared with uninfected mice (day 0).

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. 5*I*). In striking contrast, CCL2 overexpressing mice showed much higher numbers of alveolar macrophages from day 2 to 7 of infection with *S. pneumoniae* (Fig. 5*I*). In addition, CCL2 overexpressing mice showed a significantly increased number of elicited mononuclear phagocyte subsets in their lungs upon infection with *S. pneumoniae* at days 1–14 postinfection (Fig. 5*K*). These data show that 1) CCL2 overexpressing mice demonstrate a qualitatively and quantitatively different lung mononuclear phagocyte subset recruitment profile to pneumococcal challenge compared with wild-type mice, and 2) CCL2 overexpressing mice are largely protected from pneumococci-induced depletion of alveolar macrophages.

CCL2 overexpressing mice respond to challenge with 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 *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. 6*A*, *left*). Morphometric analysis of the respective lung tissue sections revealed a significantly increased number of obliterated terminal bronchioles in *S. pneumoniae*-infected CCL2 overexpressing, as compared with wild-type mice, by day 7 postinfection (Fig. 6*B*). 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

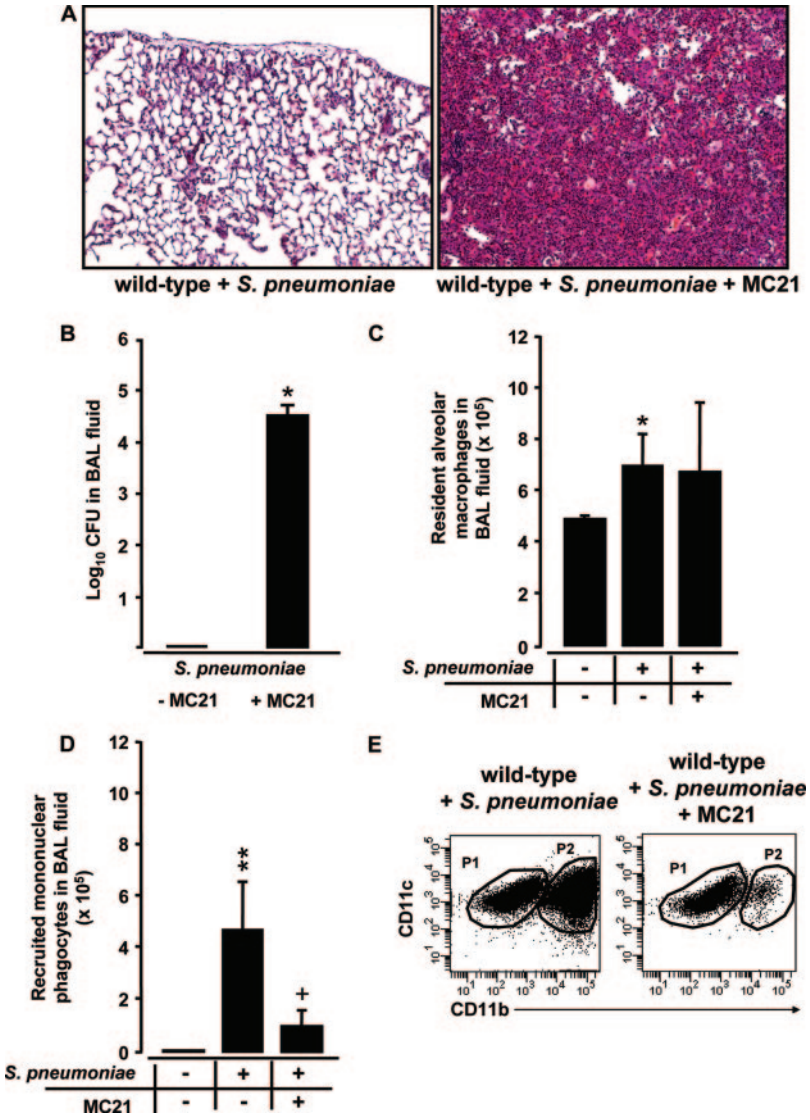

FIGURE 7. Effect of CCR2 blockade on lung histopathology, pneumococcal clearance, and mononuclear phagocyte mobilization in *S. pneumoniae*-infected wild-type mice. *A*, Lung histopathology of wild-type mice infected with *S. pneumoniae* for 7 days either in the absence (*left*) or the presence (*right*) of anti-CCR2 Ab MC21. *B*, Pneumococcal load in whole lung washes of wild-type mice at day 7 postinfection in the absence or presence of MC21 application. $*$, $p < 0.05$. Total cell number of alveolar macrophages (*C*) or recruited mononuclear phagocytes (*D*) of wild-type mice infected with *S. pneumoniae* either in the absence or presence of MC21 for 7 days. $*$, $p < 0.05$; $**$, $p < 0.01$ compared with untreated mice. $+$, $p < 0.05$ compared with *S. pneumoniae*-infected mice. *E*, Representative FACS dot plot illustrating the effect of MC21 application on the mobilization of mononuclear phagocytes in wild-type mice analyzed at day 7 postchallenge with pneumococcal infection. Data shown are mean ± SD of six mice per group and time point.

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. 6*A*, *right*) together with an impaired pneumococcal clearance (Fig. 6, *C–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 $CD11b^{pos}/CD11c^{pos}$ macrophages (Fig. 7*A*, *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 $Gr\text{-}1^{pos}$ and $Gr\text{-}1^{neg}$ monocytes. Both subsets are highly CD11b-positive, but lack CD11c, exhibit a differential CCR2 and CX3CR1 expression profile, and have the plasticity to differentiate into macrophages and/or DC (5, 16–18). In the current study, FACS-based immunophenotypic analysis showed that human CCL2 overexpression in the lungs of mice induced qualitative and quantitative differences in mononuclear phagocyte subset compositions in the transgenic mice as compared with wild-type mice. In the transgenic mice, the currently used immunophenotypic analysis

allowed us to differentiate elicited monocytic 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 $CD11b^{pos}$ and $Gr\text{-}1^{pos}$ but $CD11c^{neg}$ and $MHC\text{-}II^{neg\text{-}low}$ 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 *S. pneumoniae* 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 *S. pneumoniae*, 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 liposomal clodronate did not affect the early bacterial pathogen elimination process in mice challenged with *S. pneumoniae* (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 *S. pneumoniae* 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 *S. pneumoniae*.

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 *S. pneumoniae* known to rapidly progress to invasive pneumococcal disease. In contrast, our study infected mice via intratracheal application of capsular group 19 *S. pneumoniae* 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 *S. pneumoniae*. 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 *S. pneumoniae*-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 *S. pneumoniae*-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 *Listeria monocytogenes* (22, 23). However, the role of inflammatory elicited monocytic 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 chemotaxis 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 *S. pneumoniae* would result in stronger mononuclear phagocyte migratory responses. Importantly, MC21 treatment of *S. pneumoniae*-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 *S. pneumoniae* 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 fibroblastic 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, because 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 CCR2-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

The authors have no financial conflict of interest.

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Anlage 3

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FMS-Like Tyrosine Kinase 3 Ligand Aggravates the Lung Inflammatory Response to *Streptococcus pneumoniae* Infection in Mice: Role of Dendritic Cells[1]

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Pretreatment of mice with the hemopoietic growth factor, FMS-like tyrosine kinase 3 ligand (Flt3L), has been shown to increase monocyte-derived myeloid dendritic cells (DC) in lung parenchymal tissue, with possible implications for protective immunity to lung bacterial infections. However, whether Flt3L treatment improves lung innate immunity of mice to challenge with *Streptococcus pneumoniae* has not been investigated previously. Mice pretreated with Flt3L exhibited a peripheral monocytosis and a strongly expanded lung myeloid DC pool, but responded with a similar proinflammatory cytokine release (TNF-α, IL-6, keratinocyte derived cytokine, MIP-2, CCL2) and neutrophilic alveolitis upon infection with *S. pneumoniae* as did control mice with a normal lung DC pool. Unexpectedly, however, Flt3L-pretreated mice, but not control mice, infected with *S. pneumoniae* developed vasculitis and increased lung permeability by days 2–3 postinfection, and florid pneumonia accompanied by sustained increased bacterial loads by days 3–4 postinfection. This was associated with an overall increased mortality of ~35% by day 4 after pneumococcal challenge. Application of anti-CCR2 Ab MC21 to block inflammatory monocyte-dependent lung mononuclear phagocyte mobilization significantly reduced the lung leakage, but not vasculitis in Flt3L-pretreated mice infected with *S. pneumoniae*, without affecting the intra-alveolar cytokine liberation or the concomitantly developing neutrophilic alveolitis. Together, the data demonstrate that previous Flt3L-induced lung DC accumulation is not protective in lung innate immunity to challenge with *S. pneumoniae*, and support the concept that CCR2-dependent mononuclear phagocyte as opposed to neutrophil recruitment contributes to increased lung leakage in Flt3L-pretreated mice challenged with *S. pneumoniae*. *The Journal of Immunology,* 2007, 179: 3099–3108.

The FMS-like tyrosine kinase 3 ligand (Flt3L)[3] is a hemopoietic growth factor involved in the proliferation and differentiation of hemopoietic progenitor cells in the bone marrow. Previous reports showed that pretreatment of mice with human rFlt3L increased numbers of peripheral blood monocytes and elicited a strong mobilization and expansion particularly of myeloid dendritic cell (DC) subsets in lymphoid and nonlymphoid tissues, including the lung (1). DC reside in most tissues in an immature state, where they sample invading pathogens and other Ags and process and present them to T cells in draining lymph nodes. At the same time, DC are able to release proinflammatory cytokines, thereby participating in acute inflammatory responses (2). In chronic bacterial infections elicited by intracellular pathogens such as *Mycobacterium tuberculosis* or *Listeria monocytogenes*, application of Flt3L to increase numbers of mature, functionally active DC pools to promote induction and maintenance of adaptive immune responses has led to controversial results. One study reported that Flt3L pretreatment of mice improved protective immunity to *L. monocytogenes* in the liver (3). Another study showed that Flt3L-dependent increases in DC numbers impaired protective immunity to both *L. monocytogenes* and *M. tuberculosis* in liver and spleen, suggesting that increased DC numbers might act as reservoirs for invading pathogens (4).

Streptococcus pneumoniae is the most prevalent pathogen causing community-acquired pneumonia, septic meningitis, and otitis media worldwide (5). *S. pneumoniae*-derived cytotoxic virulence factors such as pneumolysin are known to induce apoptosis in lung sentinel cells such as alveolar macrophages, and at the same time exert strong cytotoxicity toward alveolar epithelial cells, thereby causing severe lung edema and invasive pneumococcal disease (6, 7). We most recently showed that inflammatory mononuclear phagocyte mobilization is critical to the resolution/repair phase in mice infected with *S. pneumoniae* (8). Consequently, experimentally increased numbers of mononuclear phagocyte subsets, including myeloid DC in the lungs of mice, improved protective innate immunity to challenge with *S. pneumoniae* (9). However, no data are currently available that specifically addressed the role of lung myeloid DC in the pathogenesis of pneumococcal lung infection. Because in the lung DC are situated in close vicinity to both capillary endothelial and alveolar epithelial cells, where they form an interdigitating network of sentinel cells specialized to sample inhaled bacterial pathogens (10, 11), it appears conceivable that inhaled pneumococci would have to overcome DC-based immunosurveillance of the lung to cause invasive disease progression.

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[3] Abbreviations used in this paper: Flt3L, FMS-like tyrosine kinase 3 ligand; BAL, bronchoalveolar lavage; DC, dendritic cell; FSC, forward light scatter; KC, keratinocyte derived cytokine; SSC, side light scatter.

Therefore, in the current study, we hypothesized that mice exhibiting a pre-enriched DC pool in their lungs would respond with an improved protective innate immunity to challenge with *S. pneumoniae.* To test this hypothesis, mice were pretreated with human rFlt3L to increase their lung DC pool size, and were then infected intratracheally with *S. pneumoniae*.

Materials and Methods

Mice

BALB/c mice used in the current study were purchased from Charles River Laboratories and were kept under conventional conditions with free access to food and water. Mice were used in all experiments at 8–12 wk of age in accordance with the guidelines of our Institutional Animal Care and Use Committee. Animal experiments were approved by our local government authorities.

Reagents

Abs used for FACS-based leukocyte subset-specific analysis were purchased from BD Biosciences or Serotec. In selected experiments, the rat anti-mouse anti-CCR2 mAb MC21 was used to block inflammatory mononuclear phagocyte immigration into the lungs of *S. pneumoniae*-infected mice (50 μg/mouse/day) (12, 13). Flt3L was a gift from Amgen (Thousand Oaks, CA).

Culture and quantification of S. pneumoniae

For infection studies, we used a pneumolysin-producing clinical isolate of capsular group 19 *S. pneumoniae* (EF3030). The bacteria were grown in Todd-Hewitt broth (Difco) supplemented with 0.1% yeast extract to mid-log phase, and aliquots were snap frozen in liquid nitrogen and stored at −80°C until use, as outlined in detail recently (8, 9, 14). For quantification of pneumococci, serial dilutions of the bacteria were plated on sheep blood agar (BD Biosciences) and incubated at 37°C/5% CO_2 for 18 h, followed by the determination of CFU.

Application of Flt3L and infection of mice with S. pneumoniae

Mice were either pretreated with s.c. injections of Flt3L (10 μg/mouse) or pretreated with vehicle (saline) for 9 consecutive days. Subsequently, Flt3L- or vehicle-pretreated mice were infected with *S. pneumoniae* EF3030 using freshly prepared dilutions of thawed aliquots adjusted to $\sim 2.5 \times 10^7$ CFU/mouse, as recently described (8). Briefly, tracheas were exposed by surgical resection, and intratracheal instillation of the pneumococci was performed under stereomicroscopic control (Leica Microsystems MS 5) under a laminar flow hood using a 26-gauge catheter (Abbocath; Abbott Laboratories) inserted into the trachea. After instillation, the neck wound was closed with sterile sutures, and 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.

Bronchoalveolar lavage (BAL) and lung tissue homogenization for the determination of bacterial loads in the lungs of Flt3L-pretreated and control mice challenged with S. pneumoniae

Bacterial loads within the lungs of *S. pneumoniae*-infected Flt3L-pretreated and control mice were determined from both whole lung washes and lung tissue homogenates. Briefly, mice were euthanized with an overdose of isoflurane (Forene; Abbott Laboratories), and tracheas of the mice were exposed and cannulated with a shortened 20G needle that was firmly fixed to the trachea. Subsequently, 300-μl aliquots of ice-cold sterile PBS were instilled, followed by careful aspiration until a 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 fluids (whole lung washes) collected from uninfected or *S. pneumoniae*-infected mice with or without Flt3L pretreatment 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% CO_2 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 total numbers 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. Subsequent to BAL, lung and spleen tissue, respectively, were homogenized in 2 ml of HBSS without supplements using a tissue homogenizer (IKA, Staufen, Germany), and 10-fold serial dilutions of lung and

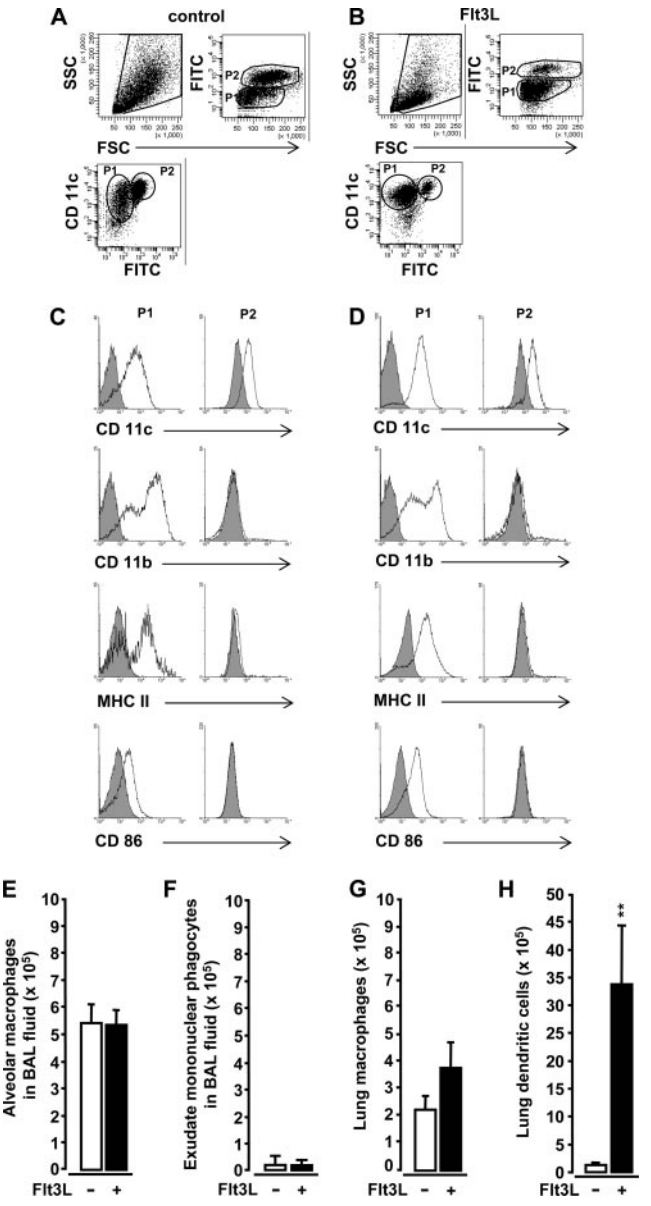

FIGURE 1. Flt3L expands the pool of myeloid DC in lung parenchymal tissue of mice. Mice either were treated with saline (*A* and *C*; □ in *E–H*) or were pretreated with Flt3L (10 μg/mouse, s.c.) for 9 consecutive days (*B* and *D*; ■ in *E–H*). Subsequently, mice were euthanized, BAL was performed, and CD11c-positive cells were purified from lung parenchymal tissue digests, as described in *Materials and Methods*. *A* and *B*, Representative flow cytometry dot plots of MACS-purified CD11c-positive lung mononuclear phagocyte subsets. Cells were gated according to their FSC vs SSC characteristics, followed by hierarchical subgating using their FSC vs green autofluorescence (FITC) properties. Gated cells (P1, P2) in *A* and *B* represent either low green autofluorescent, CD11c-positive lung myeloid DC (P1), or strongly green autofluorescent lung macrophages (P2) of control mice (*left dot plots*) or Flt3L-pretreated mice (*right dot plots*). *C* and *D*, Gated cells (P1, P2) of control mice (*C*) or Flt3L-pretreated mice (*D*) were further subjected to immunophenotypic analysis of their CD11c, CD11b, MHCII, and CD86 cell surface Ag expression profiles, as indicated. *E–H*, FACS-based quantification of alveolar macrophages (*E*), exudate mononuclear phagocytes (*F*), lung macrophages (*G*), or lung DC (*H*) collected by either BAL or MACS enrichment of CD11c-positive mononuclear phagocytes of lung tissue digests of either vehicle- or Flt3L-pretreated mice. Values are shown as mean ± SD of $n = 6$ mice per treatment group. **, Indicates significant increase ($p < 0.01$) compared with vehicle-treated control mice.

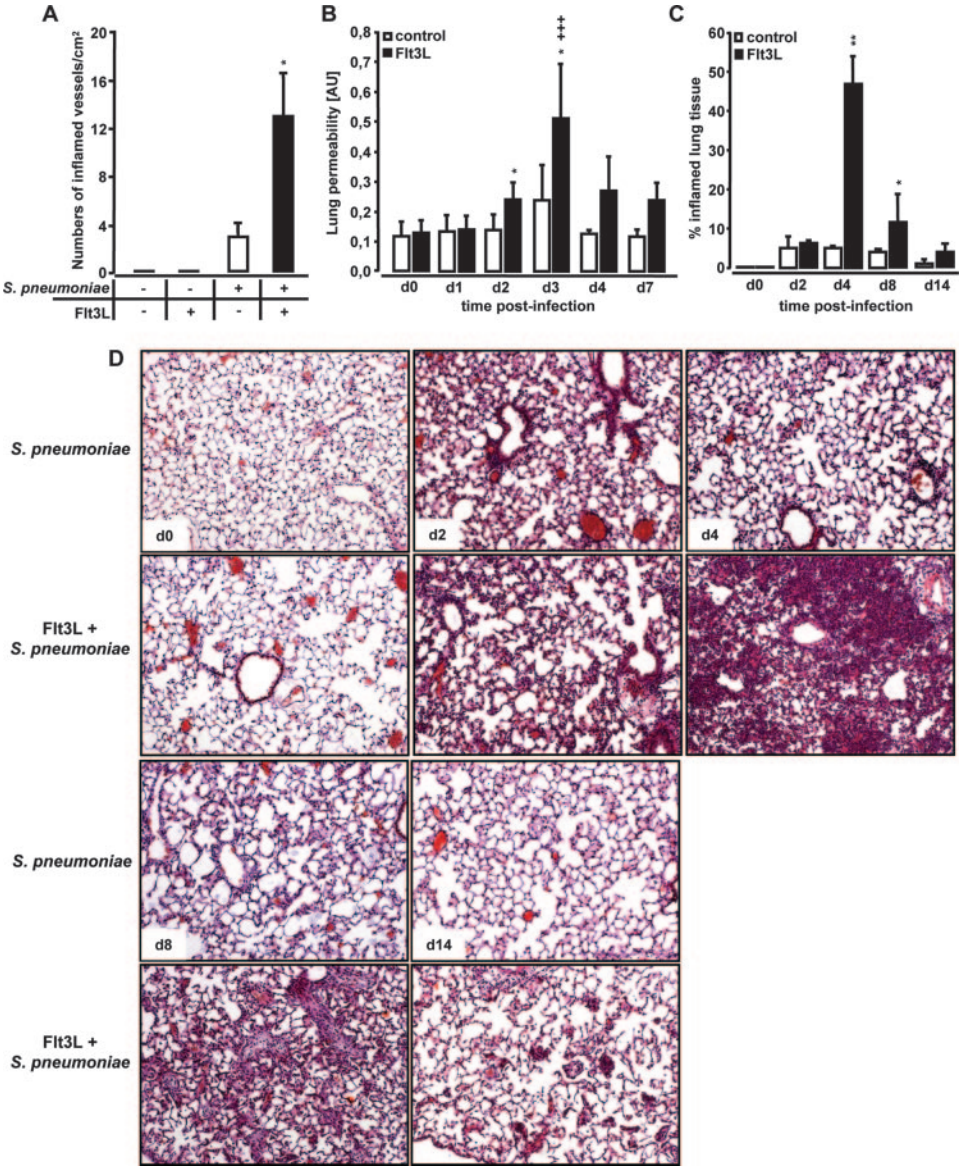

FIGURE 2. Flt3L pretreatment elicits vasculitis and lung permeability in mice challenged with *S. pneumoniae*. Mice were either vehicle treated or pretreated with Flt3L for 9 consecutive days, followed by intratracheal application of *S. pneumoniae* for the indicated time points. *A*, At day 2 postinfection, mice of the respective treatment groups were euthanized and their lungs were removed for morphometric analysis of early developing lung vascular injury. *B*, One hour before sacrifice, vehicle- or Flt3L-pretreated mice either left uninfected (d0) or infected with *S. pneumoniae* for various time points received i.v. injection of FITC-labeled human albumin (1 mg/mouse for 1 h), followed by fluorometric determination of lung permeability changes, as described in *Materials and Methods*. *C*, Degree of inflamed lung tissue in lung sections of vehicle-treated or Flt3L-pretreated mice either left uninfected (d0) or infected with *S. pneumoniae* for the indicated time points. *D*, H&E-stained histopathology of lungs collected from vehicle-treated or Flt3L-pretreated mice infected with *S. pneumoniae* for 0 , 2, 4, 8, and 14 days, as indicated. Photomicrographs are taken at a ×10 original magnification. ∗, Indicates significant difference ($p < 0.05$; ∗∗, $p < 0.01$) compared with vehicle-treated control mice infected with *S. pneumoniae*. +++, Indicates significant increase ($p < 0.001$) compared with the respective control treatment group (d0). AU, arbitrary units.

spleen tissue homogenates were plated on sheep blood agar plates, followed by incubation of the plates at 37°C/5% CO_2 for 18 h and subsequent determination of the CFU.

Quantification and characterization of peripheral blood monocytes from Flt3L-pretreated and control mice

Anticoagulated peripheral blood was collected from vehicle-treated and Flt3L-pretreated mice from the inferior vena cava. Thoroughly mixed peripheral blood was used for preparation of Pappenheim-stained blood smears, and defined volumes of peripheral blood (500 μl per mouse) were subjected to two cycles of RBC lysis in ammonium chloride solution (2 × 5 min, room temperature) and a subsequent determination of total leukocyte counts. Numbers of peripheral blood monocytes in vehicle-treated or Flt3L-pretreated mice were calculated by determining the percentage of monocyte counts in Pappenheim-stained peripheral blood smear preparations and multiplication of those values with the respective white blood cell counts. FACS analysis of peripheral blood monocytes was done by staining blood leukocyte samples with PE-conjugated anti-CD115 and allophycocyanin-conjugated F4/80 Abs, and PerCP-Cy5.5-conjugated anti-Gr-1 Abs together with secondary FITC-labeled anti-CCR2 Ab MC21.

Quantification of alveolar and lung leukocyte subsets

The quantification of alveolar recruited neutrophils contained in BAL fluids of vehicle-treated or Flt3L-pretreated mice either left uninfected or infected with *S. pneumoniae* 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. Quantification of resident and recruited mononuclear phagocyte subsets contained in BAL fluids recovered from the lungs of control mice and Flt3L-pretreated mice in the absence or presence of pneumococcal infection (BAL fluid alveolar macrophages and exudate mononuclear phagocytes) was done using FACS-based differences in cell surface Ag expression profiles (CD11b, CD11c, F4/80, MHCII, CD86), as outlined in detail recently (8, 9, 15).

Quantification of mononuclear phagocyte subsets in lung parenchymal tissue of uninfected as well as *S. pneumoniae*-infected Flt3L-pretreated and vehicle-treated mice was done according to recently published protocols (8, 9). Briefly, after BAL, lungs were carefully perfused in situ via the right ventricle with HBSS until lung lobes were visually free of blood. Subsequently, 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 DNase I for 90 min at 37°C. A further disruption of the tissue was done by pipetting with a 1-ml syringe and then passing the lung digests through 100 and 40 μM cell strainers (BD Biosciences), upon which digestion was stopped by adding RPMI 1640/10% FCS. CD11c-positive leukocyte subsets including lung macrophages and lung myeloid DC contained in lung homogenates were further purified using a CD11c 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 (Octapharma) (10 μl of octagam/10^7 cells) on ice for 10 min to block nonspecific Ab binding, and then washed with MACS buffer and incubated with CD11c 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 CD11c-positive cells (~90% purity). Purified CD11c-positive cells from lung parenchymal tissue digests were then subjected to FACS analysis of differential autofluorescence and cell surface Ag expression profiles, as outlined below, and percentages of mononuclear phagocyte subsets were multiplied by the respective total cell counts of purified CD11c-positive cells.

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

Mononuclear phagocyte subset populations contained in the lung parenchymal tissue of uninfected or *S. pneumoniae*-challenged control mice and Flt3L-pretreated 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 with specificities for the following cell surface molecules: PE-Cy7-conjugated anti-CD11b, PE-Cy5.5-conjugated anti-CD11c, PE-conjugated anti-CD86, PE-conjugated anti-MHC class II Ab (all from BD Biosciences), and allophycocyanin-conjugated anti-F4/80 (Serotec), as recently described (15). Subsequently, cells were washed in PBS/0.1% BSA/0.02% Na-azide, and cell acquisition was performed on a BD FACS Canto 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 excitation 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 green autofluorescence characteristics, followed by hierarchical subgating according to the differential CD11c vs green autofluorescence characteristics (16). Data analysis and careful postacquisition compensation of spectral overlaps between the various fluorescence channels were performed using BD FACSDiva software (BD Biosciences).

Lung permeability assay

To analyze the effect of Flt3L pretreatment on induction of lung permeability in mice challenged with *S. pneumoniae*, mice received an i.v. injection of FITC-labeled human albumin (1 mg/mouse in 100 μl of saline) (Sigma-Aldrich) 1 h before mice were euthanized by isoflurane. Undiluted BAL fluid samples and serum samples (diluted 1/100 in saline) were placed in a 96-well microtiter plate, and fluorescence intensities were measured using a fluorescence spectrometer (FL 880 microplate fluorescence reader; Bio-Tek Instruments) operating at 488 nm absorbance and 525 ± 20 nm emission wavelengths. The lung permeability index is defined as the ratio of fluorescence signals of undiluted BAL fluid samples to fluorescence signals of 1/100 diluted serum samples (12).

Lung histopathology

Vehicle-treated mice and Flt3L-pretreated mice either were left uninfected or were 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. Lung tissue samples were then paraffin embedded, and lung sections of 5 μm were stained with H&E and Elastica-van-Gieson and examined histopathologically using a Zeiss Axiovert 200 M microscope (Zeiss).

The quantification of vasculitis was done by determining total areas of two sections of the completely embedded lung specimen of either control mice or Flt3L-pretreated mice of the respective treatment groups infected with *S. pneumoniae* by planimetry. Subsequently, numbers of cross-sections of vessels fulfilling the criteria for vasculitis were counted and expressed per square centimeter of lung tissue. Vasculitis is defined as neutrophilic infiltration of the vessel wall with karyorectic debris and at least segmental necrosis of the medium, sometimes with associated fibrin thrombi.

ELISA

Proinflammatory cytokine release in BAL fluids of uninfected or *S. pneumoniae*-infected control mice and Flt3L-pretreated mice was determined using commercially available ELISA (R&D Systems).

Statistics

All data are given as mean ± SD. Differences between untreated and Flt3L-pretreated mice infected with *S. pneumoniae* were analyzed by ANOVA, followed by post hoc Dunnett test. Differences between groups of *S. pneumoniae*-infected mice pretreated with Flt3L in the absence or presence of MC21 were analyzed by ANOVA, followed by post hoc Scheffe test. 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 groups were assumed when p values were <0.05.

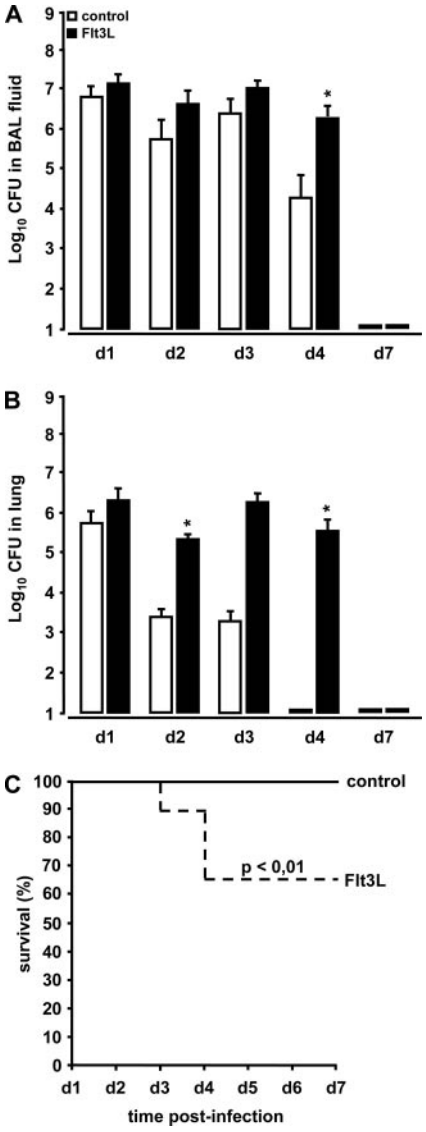

FIGURE 3. Bacterial loads in lung washes and lung tissue homogenates, and survival in Flt3L-pretreated and control mice in response to infection with *S. pneumoniae*. *A*, Bacterial loads in whole lung washes and lung tissue homogenates (*B*) of either vehicle-treated (□) or Flt3L-pretreated mice (■) infected with *S. pneumoniae* for various time intervals, as indicated. *C*, Survival of vehicle-treated or Flt3L-pretreated mice after intratracheal infection with *S. pneumoniae* for the observation period of 7 days. Values are shown as mean ± SD of $n = 6$ mice (*A*), $n = 6$ mice (*B*), and $n = 18$ mice (*C*) per treatment group and time point. *, Indicates significant difference ($p < 0.05$; ***, $p < 0.001$) compared with vehicle-treated control mice infected with *S. pneumoniae*.

Results

Flt3L pretreatment of mice elicits increased numbers of circulating monocytes and lung myeloid DC

We initially analyzed the effect of Flt3L pretreatment on circulating monocyte and neutrophil counts and the lung myeloid DC pool

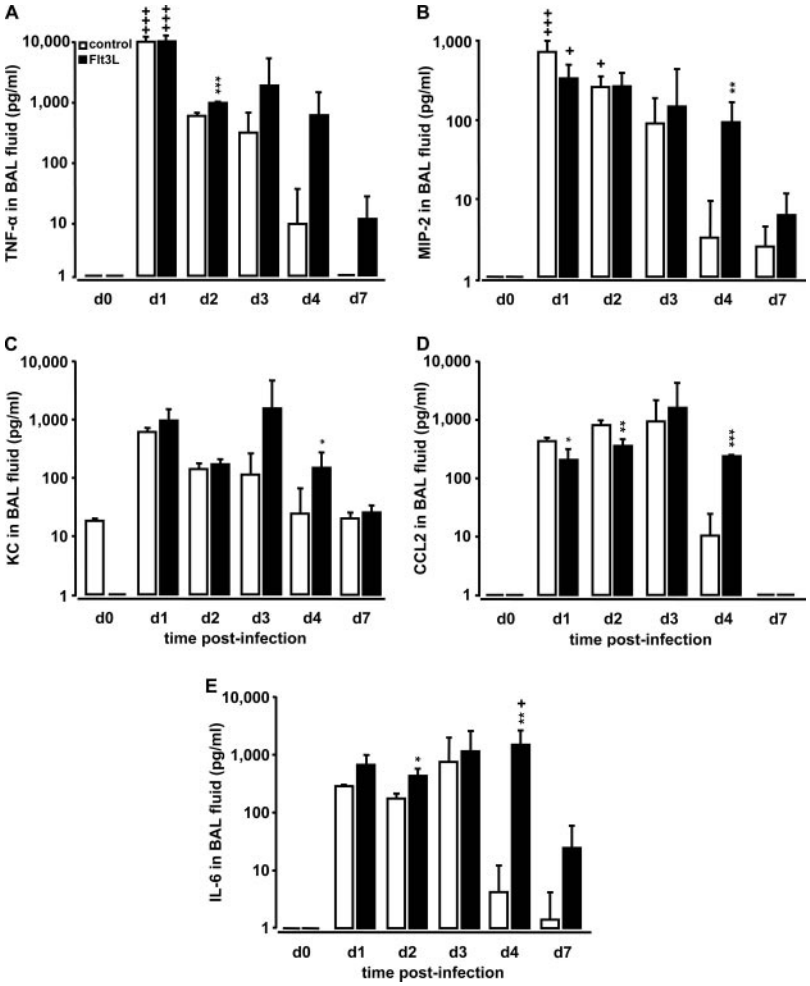

FIGURE 4. Bronchoalveolar cytokine/chemokine responses in Flt3L-pretreated as compared with control mice challenged with *S. pneumoniae*. Mice were either vehicle pretreated (□) or Flt3L pretreated (■), followed by infection with *S. pneumoniae* for 1, 2, 3, 4, and 7 days, as indicated. Subsequently, mice were euthanized and subjected to BAL for the quantification of TNF-α (*A*), MIP-2 (*B*), KC (*C*), CCL2 (*D*), or IL-6 (*E*). Values are shown as mean ± SD of $n = 8$ mice per time point and treatment group. +, Indicates significant difference ($p < 0.05$; +++, $p < 0.001$) compared with the respective control group (d0). ∗, Indicates significant increase/decrease ($p < 0.05$; ∗∗, $p < 0.01$; ∗∗∗, $p < 0.001$) compared with vehicle-treated, *S. pneumoniae*-infected mice.

size in uninfected mice. Vehicle-treated mice were found to have $1.1 \pm 0.4 \times 10^2$ monocytes and $5.1 \pm 2.4 \times 10^2$ neutrophils per μl of peripheral blood, whereas Flt3L-pretreated mice had $1.2 \pm 0.4 \times 10^3$ monocytes ($n = 4$; $p < 0.02$) and $1.3 \pm 0.8 \times 10^3$ neutrophils per μl of peripheral blood ($n = 4$; NS). Moreover, as shown in Fig. 1, FACS-based characterization of CD11c-positive cells purified from lung parenchymal tissue of vehicle-treated or Flt3L-pretreated mice yielded two major mononuclear phagocyte subsets (P1, P2), as defined by their differential green autofluorescence properties in conjunction with cell surface Ag expression profiles of CD11c, CD11b, MHCII, and CD86, corresponding to recent reports (16). CD11c-positive cells collected from the lung parenchymal tissue of control mice according to their differential autofluorescence and immunophenotype were mainly composed of lung macrophages (P2; Fig. 1*A*) and, to a lesser extent, of lung myeloid DC (P1; Fig. 1, *A*, *G*, and *H*). FACS analysis of these two major mononuclear phagocyte subsets showed that lung macrophages were green autofluorescent and $CD11c^+$, $CD11b^-$, $MHCII^{-/low}$, and $CD86^-$, whereas lung myeloid DC lacked green autofluorescence, but were found to be $CD11c^+$ and $CD11b^+$, $MHCII^{high}$, and $CD86^+$ (P1; Fig. 1, *A* and *C*). Of note, Flt3L-pretreated mice had a significantly (~14-fold) increased lung myeloid DC pool size with a similar immunophenotypic profile, compared with lung DC of vehicle-treated control mice (P1; Fig. 1, *B*, *D*, and *H*). At the same time, numbers of lung macrophages did not differ significantly between Flt3L-pretreated as compared with vehicle-treated control mice (P2; Fig. 1, *A*, *B*, and *G*). Also, Flt3L pretreatment of mice did not affect numbers of resident alveolar macrophages or alveolar exudate mononuclear phagocytes compared with vehicle-treated controls (Fig. 1, *E* and *F*). Finally, both vehicle-treated and Flt3L-pretreated mice had a small, but detectable portion of plasmacytoid DC ($CD11c^{low-mid}$, $CD11b^-$, $B220^+$) in their lung homogenates (data not shown).

Flt3L application elicits vasculitis and lung permeability changes in mice challenged with S. pneumoniae

Initial dose-response experiments revealed that intratracheal challenge of BALB/c mice with $\sim 3 \times 10^7$ CFU *S. pneumoniae* provoked an increased mortality of infected mice of ~50% by day 3 postinfection (data not shown) (9). Therefore, in the current study, *S. pneumoniae* infection doses were adjusted to $\sim 2.5 \times 10^7$ CFU *S. pneumoniae*/mouse.

Mice pretreated with Flt3L responded with progressive acute lung injury upon infection with *S. pneumoniae* (2.5×10^7 CFU/mouse), as characterized by a rapidly developing, significantly increased vasculitis and microthrombus formation (Fig. 2*A*), and significantly increased lung permeability at day 2, peaking at day 3 postinfection, with a further decline by day 7 postinfection (Fig. 2*B*). As shown in Fig. 2, *C* and *D*, H&E-stained lung tissue sections of Flt3L-pretreated mice challenged with *S. pneumoniae* revealed confluent bronchopneumonia by day 2 postinfection, progressing into lobar pneumococcal pneumonia with a significantly increased degree of inflamed lung tissue of ~50% by day 4 postinfection, and resolving thereafter by day 14

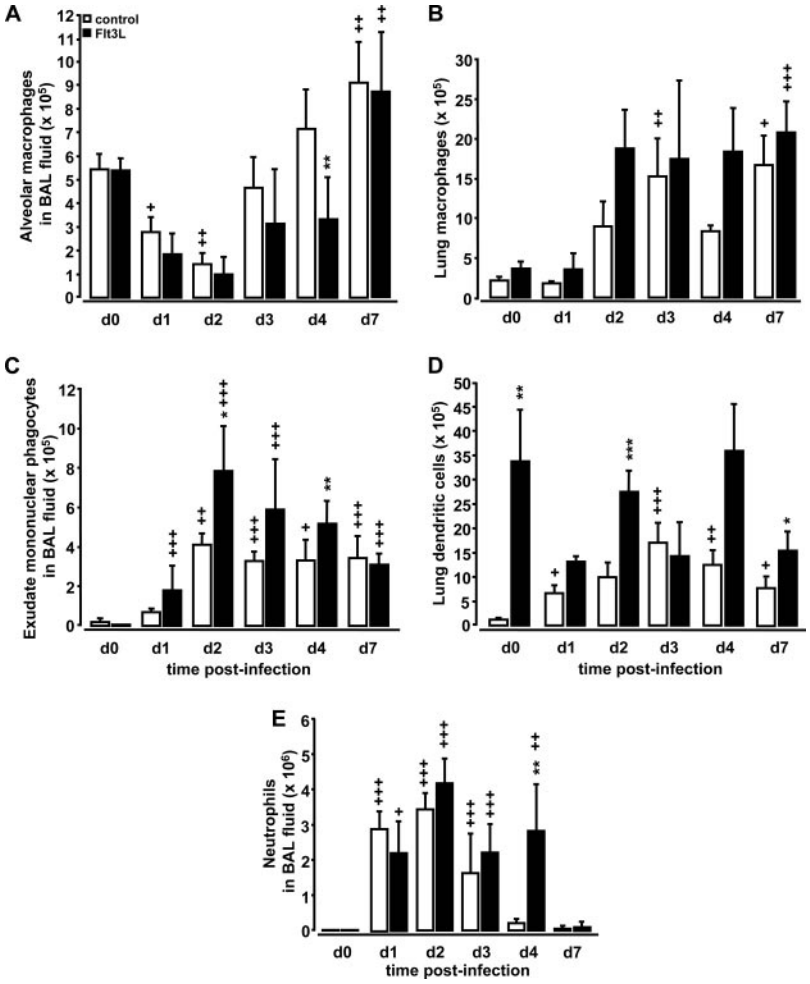

FIGURE 5. Leukocyte subset recruitment kinetics in Flt3L-pretreated and control mice challenged with *S. pneumoniae*. Vehicle-treated (□) or Flt3L-pretreated (■) mice either were left uninfected (day 0 time points) or were infected intratracheally with *S. pneumoniae* for 1, 2, 3, 4, or 7 days, as indicated. Subsequently, mice were euthanized and subjected to BAL for the quantification of BAL fluid alveolar macrophages (*A*), exudate macrophages (*C*), or neutrophils (*E*), respectively. CD11c-positive lung macrophages (*B*) and CD11c-positive lung DC (*D*) were purified from lung parenchymal tissue collected from mice of the respective treatment groups and quantified, as outlined in *Materials and Methods*. Values are shown as mean ± SD of $n = 8$ mice (*A*, *C*, and *E*) and $n = 6$ mice (*B* and *D*) per time point and treatment group. +, Indicates significant increase/decrease ($p < 0.05$; ++, $p < 0.01$; +++, $p < 0.001$) compared with the respective control groups (d0). ∗, Indicates significant increase/decrease ($p < 0.05$; ∗∗, $p < 0.01$; ∗∗∗, $p < 0.001$) compared with vehicle-treated, *S. pneumoniae*-infected mice.

postinfection (Fig. 2, *C* and *D*). In contrast, control mice infected with 2.5×10^7 CFU *S. pneumoniae* did not develop increased vasculitis or lung permeability (Fig. 2, *A* and *B*) and showed a mild bronchopneumonia not progressing toward lobar pneumonia with an overall inflamed lung tissue not exceeding ~10% with a restitutio ad integrum observed by day 8 postinfection (Fig. 2, *C* and *D*).

Bacterial loads in lung washes (Fig. 3*A*) and lung tissue homogenates (Fig. 3*B*) of vehicle-treated control mice infected with *S. pneumoniae* decreased over time, and bacteria were significantly decreased in lung washes (day 4) and undetectable in lung tissue by days 4 and 7 postinfection. At the same time, Flt3L-pretreated mice failed to purge pneumococcal infection during the first 4 days of infection, at which time bacterial loads in both whole lung washes and lung tissue homogenates were still significantly elevated when compared with *S. pneumoniae*-challenged control mice (Fig. 3, *A* and *B*). Most importantly, by day 4 postinfection, ~35% of the Flt3L-pretreated mice, but none of the control mice succumbed to pneumococcal pneumonia (Fig. 3*C*), collectively demonstrating that Flt3L pretreatment of mice results in a decreased bacterial pathogen elimination process and a severely aggravated lung inflammatory response to Gram-positive bacterial infection. To further investigate whether the increased lung permeability observed in the Flt3L-pretreated mice may have caused septic disease progression in these mice subsequent to pneumococcal infection, we determined bacterial loads in whole blood and spleen homogenates from mice of either treatment group during the observation period of 7 days. Interestingly, none of the investigated mice of either treatment group was found to demonstrate septic disease progression during the observation period, thus excluding sepsis induction as the underlying reason for the decreased survival noted in the Flt3L-pretreated mice (data not shown).

Proinflammatory cytokine profiles and leukocyte recruitment patterns in S. pneumoniae*-infected mice with or without Flt3L pretreatment*

Analysis of proinflammatory mediator profiles in BAL fluids of Flt3L-pretreated and vehicle-treated, *S. pneumoniae*-infected mice revealed rapidly increased TNF-α protein levels upon infection, peaking by day 1 postinfection with visual differences between groups particularly on day 4 postinfection, although these differences did not reach statistical significance (Fig. 4*A*). Moreover, mice of either treatment group initially responded with strongly elevated neutrophil chemoattractants MIP-2 and keratinocyte derived cytokine (KC) (CXCL1/2), the monocyte chemoattractant CCL2, and the acute-phase protein IL-6, with peak levels mainly observed between day 1 up until day 3 postinfection (Fig. 4, *B–E*). Although Flt3L-pretreated mice compared with vehicle-treated mice demonstrated slightly, but significantly elevated MIP-2, KC, CCL2, and IL-6 cytokine levels at day 4 postinfection, overall profiles of cytokine responses between mice of either treatment group were largely similar during the observation period of 7 days.

We next analyzed the mononuclear phagocyte subset distribution profiles in BAL fluids and lung parenchymal tissue of Flt3L-pretreated and vehicle-treated mice in the absence or presence of

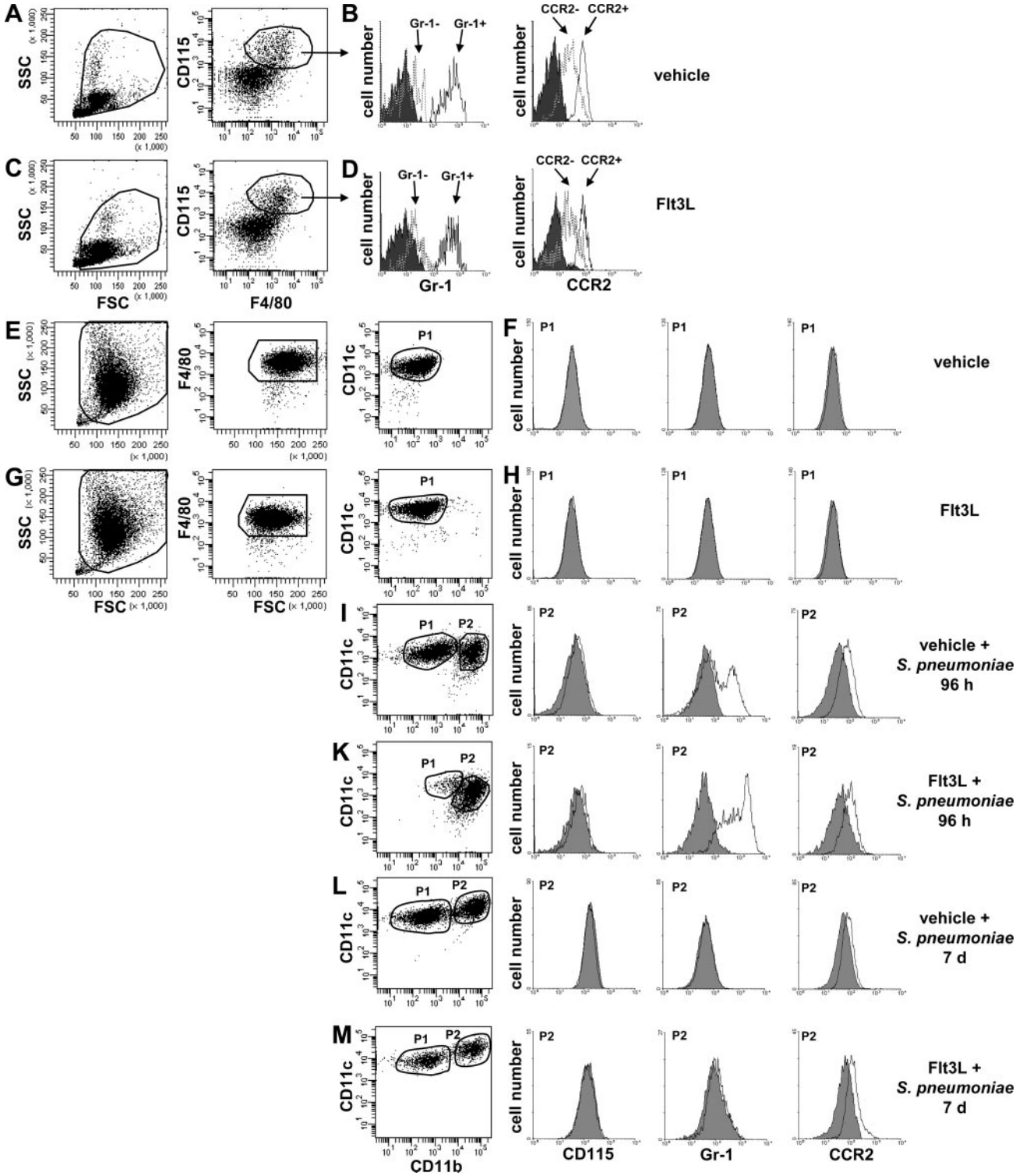

FIGURE 6. Phenotypic analysis of peripheral blood monocyte and alveolar macrophage subsets in vehicle-treated and Flt3L-pretreated mice. *A–D*, Peripheral blood leukocytes were collected from vehicle-treated (*A* and *B*) and Flt3L-pretreated (*C* and *D*) mice, as described in *Materials and Methods*. Subsequently, cells were gated according to their FSC/SSC characteristics (*A*, *left dot plot*), followed by hierarchical subgating of the CD115/F4/80 double-positive monocyte population (*B*, *left dot plot*). Subsets of Gr-1-positive monocytes (solid lines in *B* and *D*, *left histograms*) vs Gr-1-negative monocytes (dashed line in *B* and *D*, *left histograms*) were analyzed for their respective CCR2 cell surface expression (CCR2 positive: solid lines in *B* and *D*, *right histograms*, as indicated). Dashed lines in *B* and *D* represent Gr-1$^-$, CCR2$^{-/low}$ monocytes, as indicated. Filled histograms in *B* and *D* represent negative controls. Monocyte subsets represented by dashed and solid lines in *left histograms* of *B* and *D* are identical with the respective monocyte subsets represented by dashed and solid lines in *right histograms* of *B* and *D*. The shown FACS analysis is representative of three independent experiments. *E–M*, Alveolar macrophages (P1) or alveolar exudate macrophages (P2) recovered by BAL from the lungs of vehicle-treated mice or Flt3L-pretreated mice either left uninfected (*E–H*) or infected with *S. pneumoniae* (*I–M*) were gated according to their FSC/SSC characteristics as well as their FSC/F4/80 characteristics, as indicated. Subsequently, alveolar macrophages (P1) or alveolar exudate macrophages (P2) were gated according to their CD11c vs CD11b Ag expression (*E* and *G*, *right dot plots*, or *I–M*, *single dot plots*) and then analyzed for their CD115, Gr-1, or CCR2 cell surface Ag expression profile, as indicated. Filled histograms represent negative controls, and lined overlay histograms represent specific cell surface Ag expression. The shown FACS analyses are representative of three independent experiments.

pneumococcal infection. As shown in Fig. 5*A*, mice with or without Flt3L pretreatment responded with a strong and significant drop in numbers of alveolar macrophages, with lowest cell numbers observed at day 2 postinfection, which was followed by reexpansion of the alveolar macrophage pool in both treatment groups at later time points (Fig. 5*A*). At the same time, we observed that numbers of lung parenchymal macrophages from *S. pneumoniae*-infected vehicle-treated or Flt3L-pretreated mice continuously increased over time (Fig. 5*B*). FACS-based analysis of newly recruited mononuclear phagocytes in BAL fluids of vehicle-treated vs Flt3L-pretreated mice challenged with *S. pneumoniae* revealed a significantly increased alveolar accumulation of exudate macrophages ($CD11c^+$, $CD11b^+$, $MHCII^-$, $CD86^-$) peaking by day 2 postinfection and declining toward lower levels by day 7 postinfection. Analysis of lung parenchymal DC from mice of either treatment group showed that control mice infected with *S. pneumoniae* responded with a moderate, yet mostly significant increase in lung DC numbers up until day 3 of infection and a return to lower levels by day 7 postinfection (Fig. 5*D*). In contrast, Flt3L-pretreated mice with an expanded lung DC pool responded with a drop in lung DC numbers by day 1 postpneumococcal infection, followed by a continuous increase of lung parenchymal DC up until day 4 and a decline toward lower levels by day 7 postinfection (Fig. 5*D*). Importantly, whereas vehicle-treated mice infected with *S. pneumoniae* demonstrated a progressive neutrophilic alveolitis peaking by day 2 and declining toward baseline levels by day 4 postinfection, Flt3L-pretreated mice showed a sustained alveolar neutrophilic response up until day 4 and a return to baseline levels by day 7 postinfection (Fig. 5*E*), which is of particular importance given the still significantly elevated CFU counts noted in these mice by day 4 of infection (Fig. 5*E*, compare with Fig. 3, *A* and *B*).

Phenotypic analysis of peripheral blood monocyte and macrophage subsets in vehicle-treated and Flt3L-pretreated mice

It is currently unknown whether Flt3L pretreatment of mice affects phenotypes of circulating monocytes or monocyte-derived mononuclear phagocyte subsets either under baseline or inflammatory conditions in vivo, which may have possible implications for the mobilization of monocyte-derived macrophage subsets and the resolution of inflammation. FACS analysis of blood monocyte subsets of vehicle-treated and Flt3L-pretreated mice revealed that circulating monocytes of either treatment group coexpressed CD115 and F4/80 and were composed of $Gr\text{-}1^+/CCR2^+$ and $Gr\text{-}1^{-/low}/CCR2^{-/low}$ monocyte subsets (Fig. 6, *A–D*). This demonstrates that Flt3L treatment of mice elicited quantitative rather than qualitative changes in peripheral blood monocyte subset distribution profiles. Moreover, FACS analysis of alveolar macrophages collected from the lungs of vehicle-treated mice (Fig. 6, *E* and *F*) and Flt3L-pretreated mice (Fig. 6, *G* and *H*) revealed a $F4/80^+$, $CD115^-$, $CCR2^{-/low}$, and $Gr\text{-}1^-$ immunophenotypic profile of these cells, with no differences observed between groups (Fig. 6, *E–H*). A similar immunophenotypic profile was also analyzed on alveolar macrophages recovered from the lungs of both vehicle-treated and Flt3L-pretreated mice challenged with *S. pneumoniae* for either 4 or 7 days (data not shown in detail). In contrast, alveolar recruited exudate macrophages (P2; Fig. 6, *I–M*) recovered from the lungs of vehicle- or Flt3L-pretreated mice challenged with *S. pneumoniae* were found to be $CD11b^+$, $CD115^-$, $CCR2^+$, and $Gr\text{-}1^{+/middle}$ (Fig. 6, *I* and *K*, *middle histogram*), with no overt differences in immunophenotypic profiles of alveolar exudate macrophages between treatment groups. These data support the view that Flt3L pretreatment of mice does not affect phenotypic profiles of inflammatory monocytes or monocyte-derived mononuclear phagocyte subsets (alveolar macrophages, exudate macrophages), but rather elicits quantitative changes in baseline (e.g., lung DC) and inflammatory elicited lung mononuclear phagocyte subset recruitment in response to bacterial infection.

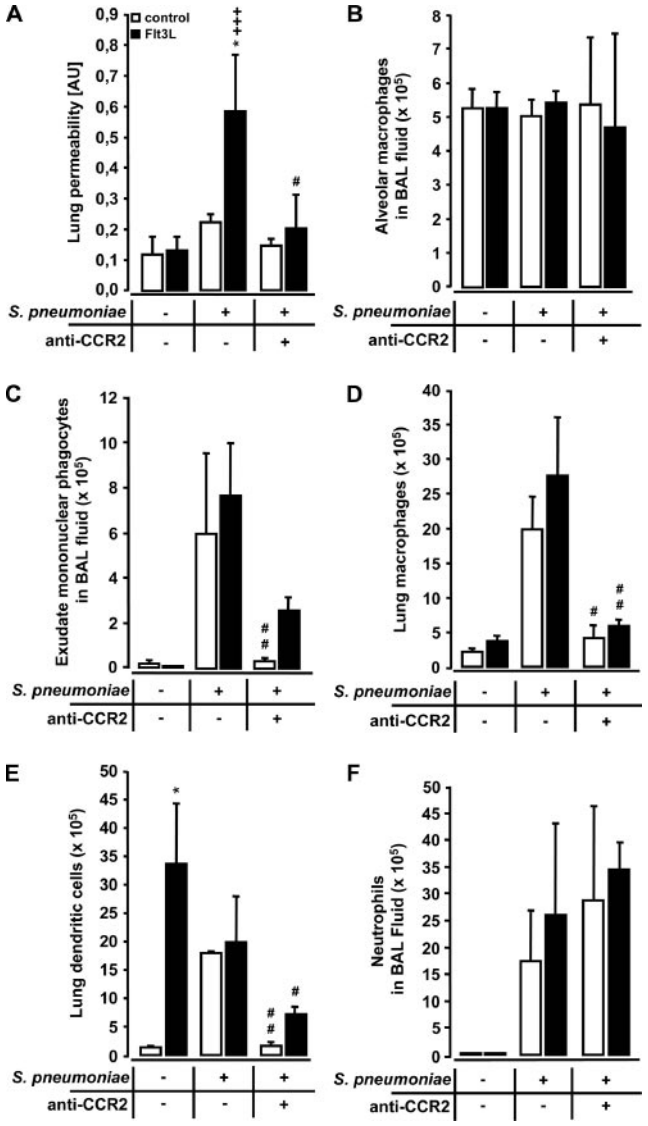

FIGURE 7. Effect of CCR2 blockade on lung permeability and inflammatory leukocyte recruitment in Flt3L-pretreated mice challenged with *S. pneumoniae*. Vehicle-treated mice (□) or Flt3L-pretreated mice (■) either were left uninfected or were infected with *S. pneumoniae* for 3 days or were pretreated with Flt3L, followed by infection with *S. pneumoniae* for 3 days in the presence of anti-CCR2 Ab MC21 (50 μg/mouse/day). *A*, One hour before sacrifice, mice received an i.v. injection of FITC-labeled human albumin (1 mg/mouse for 1 h) for fluorometric analysis of lung permeability. Numbers of alveolar macrophages (*B*), exudate macrophages (*C*), CD11c-positive lung parenchymal macrophages (*D*), and CD11c-positive lung myeloid DC (*E*) purified from lung parenchymal tissue digests of mice of the indicated treatment groups and alveolar recruited neutrophils (*F*) recovered by BAL from mice of the various treatment groups, as indicated. Values are shown as mean ± SD of $n = 6$ mice per treatment group. *, Indicates significant increase ($p < 0.05$) compared with vehicle-treated control mice. +++, Indicates significant increase ($p < 0.001$) compared with the respective Flt3L-pretreated-only control group. #, Indicates significant decrease ($p < 0.05$; ##, $p < 0.01$) compared with the respective *S. pneumoniae*-infected vehicle-treated mice or Flt3L-pretreated and *S. pneumoniae*-infected mice.

Effect of CCR2 blockade on lung permeability and inflammatory leukocyte recruitment in Flt3L-pretreated mice challenged with S. pneumoniae

We next questioned whether the significantly increased inflammatory mononuclear phagocyte subset recruitment particularly observed by day 2 postinfection accounted for the increased vasculitis and later developing lung permeability peaking by day 3 postinfection in the Flt3L-pretreated mice challenged with *S. pneumoniae*. Application of anti-CCR2 Ab MC21, which is known to particularly block inflammatory $Gr1^+/CCR2^+$ monocyte subset recruitment (12, 17), nearly completely inhibited peak lung barrier dysfunction (Fig. 7*A*), but not vasculitis (data not shown) in Flt3L-pretreated mice, which was observed to increase by day 2 and to peak by day 3 postpneumococcal challenge. Importantly, anti-CCR2 Ab application did not affect numbers of alveolar macrophages as opposed to alveolar exudate macrophages, lung macrophages, and lung DC in Flt3L-pretreated and vehicle-treated mice challenged with *S. pneumoniae* (Fig. 7, *B–E*). At the same time, numbers of alveolar recruited neutrophils were not affected by MC21 application in Flt3L-pretreated or vehicle-treated mice infected with *S. pneumoniae* for 3 days (Fig. 7*F*).

Discussion

In the current study, we tested the hypothesis that Flt3L-induced accumulation of myeloid DC in the lung parenchymal tissue of mice would increase lung protective immunity to infection with the prototype Gram-positive bacterial pathogen, *S. pneumoniae*. However, we found that Flt3L-pretreated mice responded to infection with *S. pneumoniae* with an early developing vasculitis, microthrombus formation, and lung permeability, followed by a reduced pneumococcal clearance and progressive lobar pneumonia, resulting in a significantly increased mortality of Flt3L-pretreated mice of 35% by day 4 postinfection. These data show that Flt3L pretreatment of mice does not improve, but rather decreases lung protective immunity against *S. pneumoniae* infection, thus highlighting the necessity of a tightly controlled lung mononuclear phagocyte network in protective immunity to inhaled bacterial pathogens.

Flt3L-pretreated mice challenged with *S. pneumoniae* exhibited an overall mortality of ~35% by day 4 postinfection, as opposed to vehicle-treated control mice not succumbing to pneumococcal infection during the observation period of 7 days. Importantly, this increased mortality in the Flt3L-pretreated mice was preceded by an early developing vasculitis, microthrombus formation, and increased lung permeability by days 2–3 postpneumococcal infection and an impaired capacity of the Flt3L-pretreated mice to purge the pneumococcal infection. Flt3L pretreatment of mice alone was not sufficient to elicit the reported increased vasculitis and lung permeability changes, thus clearly involving an inflammatory activation step. Importantly, whole blood and spleen tissue homogenates collected from infected mice of either treatment group during the observation period of 7 days did not provide evidence for septic disease progression in the currently described Flt3L pneumonia model, thus excluding the possibility that the decreased capacity to purge the pneumococcal infection along with the increased lung permeability observed in the Flt3L-pretreated mice may have accounted, at least in this regard, to the observed decreased survival in these mice. Moreover, proinflammatory cytokine/chemokine levels analyzed in BAL fluids of mice of the two treatment groups during the observation period of 7 days exhibited similar profiles, with only slight differences observed between groups by day 4 postinfection, thus strongly arguing against a major impact of proinflammatory mediators to directly contribute to the noted differences in outcome between treatment groups. In contrast, we found that Flt3L application triggered both a transient peripheral blood neutrophilia and monocytosis in mice, followed by a strongly increased accumulation of myeloid DC in lung parenchymal tissue, in line with recent reports (4). Therefore, it is conceivable that increased numbers of neutrophils and/or inflammatory monocytes or monocyte-derived mononuclear phagocyte subsets such as lung myeloid DC contributed to the vasculitis and/or lung permeability changes as well as decreased pneumococcal clearance capacities, with possible implications for the decreased survival noted in the Flt3L-pretreated mice challenged with *S. pneumoniae*. Of note, FACS analysis of peripheral blood monocytes collected from vehicle-treated or Flt3L-pretreated mice largely ruled out the possibility that Flt3L pretreatment of mice selectively affected monocyte subset compositions in peripheral blood. In contrast, Flt3L pretreatment strongly increased particularly numbers of lung DC, which are positioned in close vicinity to both capillary endothelium and alveolar epithelium to evoke lung permeability changes upon inflammatory activation. In addition to lung DC acting as possible culprits in the induction of lung permeability in the described model, neutrophils have been implicated as classical effector cells in acute lung injury both in murine models of lung inflammation and in human diseases (18). However, both BAL fluid levels of neutrophil chemoattractants KC and MIP-2 (CXCL1/2) and the concomitantly developing neutrophilic alveolitis observed at day 1 up until day 3 postinfection were not different in vehicle-treated as compared with Flt3L-pretreated, *S. pneumoniae*-infected mice, thus strongly arguing against a major role of inflammatory recruited neutrophils in induction of lung barrier dysfunction in the Flt3L-pretreated mice infected with *S. pneumoniae*. In contrast, application of anti-CCR2 Ab MC21 strongly inhibited the lung barrier dysfunction in mice of the Flt3L group without blocking the developing neutrophilic alveolitis or vasculitis, thus again excluding elicited neutrophils to contribute to the lung permeability observed in the current model. Anti-CCR2 Ab MC21 is known to efficiently block inflammatory monocyte recruitment toward acutely inflamed lungs (7, 12, 17, 19). Thus, application of MC21 to block CCR2 function in vivo was expected to inhibit inflammatory mobilization of the CCR2-positive rather than CCR2-negative circulating monocyte subset in Flt3L-pretreated mice upon infection with *S. pneumoniae*. Indeed, application of anti-CCR2 Ab MC21 significantly reduced the lung barrier dysfunction in Flt3L-pretreated, *S. pneumoniae*-infected mice, while at the same time strongly inhibiting numbers of monocyte-derived lung parenchymal macrophages, alveolar exudate macrophages, and particularly lung DC. These data strongly support the concept that the significantly increased lung permeability observed in Flt3L-pretreated as compared with vehicle-treated mice infected with *S. pneumoniae* is most probably due to increased inflammatory lung mononuclear phagocyte subset recruitment in these mice, as a consequence of the Flt3L pretreatment regimen, and may have contributed to the overall reduced outcome in the Flt3L treatment group. However, because inflammatory monocytes have a considerable plasticity to differentiate into lung macrophages and lung myeloid DC (20, 21), and because MC21 application selectively blocks the CCR2-dependent inflammatory monocyte recruitment and therefore derived mononuclear phagocyte subset mobilization, we are currently not able to further pinpoint the observed lung permeability changes noted in the Flt3L-pretreated mice to a specific mononuclear phagocyte subset. Collectively, the data of the current study for the first time support the concept of an important involvement of CCR2-dependent inflammatory mononuclear phagocyte interference with sessile barrier cells of the lung to provoke lung barrier dysfunction and poor outcome in Flt3L-pretreated mice infected with *S. pneumoniae*.

We recently demonstrated that global deletion or inhibition of signaling pathways with critical relevance for inflammatory mononuclear phagocyte recruitment such as represented by PI3Kγ and

CCR2 may result in detrimental effects on outcome in pneumococcal pneumonia (8, 9). In contrast, CCL2 chemokine-dependent mononuclear phagocyte mobilization toward *S. pneumoniae*-challenged lungs using CCL2-overexpressing mice improved protective immunity of the lung to challenge with *S. pneumoniae*, although at the same time eliciting fibroproliferative responses in infected lungs (9). In this context, alveolar macrophages are currently the best-characterized mononuclear phagocyte subset of the lung that mediates central steps of the host defense against inhaled bacterial pathogens, including the phagocytosis and killing of bacteria. Alveolar macrophages that undergo apoptosis in response to *S. pneumoniae* infection may contribute to enhanced bacterial killing, and additionally restrict the proinflammatory response to inhaled bacteria, thus attenuating overwhelming local and systemic proinflammatory responses to *S. pneumoniae* infection. At the same time, alveolar and exudate macrophages play important roles in the resolution/repair phase of the disease, thus serving to regain alveolar and lung homeostasis (22–26). The currently presented data, making use of a largely (yet not exclusively) DC-specific growth factor for the first time, aim at gaining further insights into the role of mononuclear phagocyte subsets other than classical alveolar macrophages in the lung host defense to *S. pneumoniae* infection and highlight the critical importance of a fine-tuned mononuclear phagocyte network within the lung for an optimal host defense to inhaled bacterial pathogens. In view of future clinical strategies aiming at improving DC-dependent adaptive immune responses to noninfectious diseases, such as metastatic lung cancer (27, 28), it will be important to determine whether these intervention strategies to modulate DC pool sizes interfere with lung inflammatory responses to pulmonary infections.

Collectively, our initial hypothesis that Flt3L pretreatment renders mice more resistant to challenge with the prototype Gram-positive bacterial pathogen, *S. pneumoniae*, was wrong. The data of the current study show that a Flt3L-induced increase in numbers of lung myeloid DC and possibly exudate macrophages may have serious consequences for the host challenged with *S. pneumoniae*, thus arguing against therapeutic interventions to modulate numbers of lung DC as a strategy to improve lung innate immunity against invading bacterial pathogens.

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Disclosures

The authors have no financial conflict of interest.

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Anlage 4

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Lack of lung mononuclear phagocyte recruitment elicits sepsis in mice infected with non-invasive *Streptococcus pneumoniae*

Manuskript zur Publikation eingereicht

Lack of lung mononuclear phagocyte recruitment elicits sepsis in mice infected with non-invasive *Streptococcus pneumoniae*

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Running title: CCL2 in pneumococcal pneumonia

Abstract (200 words)

Background: Inflammatory monocyte-derived phagocytes are critical components of lung immunity against inhaled bacterial pathogens. Their recruitment requires establishment of CCL2 chemokine gradients between lung compartments. *Streptococcus pneumoniae* is an important lung pathogen either causing non-invasive infection or bacteraemia and sepsis. **Methods and Results:** We determined whether absence of CCL2 gradients would enable otherwise non-invasive serotype 19 *S. pneumoniae* EF3030 to escape lung immune surveillance and cause septic pneumococcal disease in mice. Despite a strong alveolar neutrophil recruitment, CCL2 KO mice showed significantly higher bacterial loads in their lungs relative to wild-type mice and developed bacteraemia by day 3 post infection with *S. pneumoniae*, resulting in a significant ~55% decrease in survival by day 10 post-infection compared to 100% survival in *S. pneumoniae*-challenged wild-type mice. Such increased mortality was associated with impaired *de novo* recruitment of lung exudate macrophages and thus repopulation of the alveolar macrophage pool in the KO mice challenged with *S. pneumoniae*. **Conclusions:** Lung exudate macrophages serve as “barrier” cells to prevent non-invasive *S. pneumoniae* from escaping local immune surveillance and causing sepsis in mice. Thus, breakdown of local protective immune responses may cause otherwise harmless infections of the lung with *S. pneumoniae* to exhibit fatal disease progression.

Key words: Macrophage, monocyte, chemokine, sepsis, infection

Introduction

Streptococcus pneumoniae (the pneumococcus) is the most frequently identified pathogen in community-acquired pneumonia worldwide [1]. The pathogenicity of *S. pneumoniae* is largely defined by its arsenal of virulence factors, among which the pore-forming cytolysin, pneumolysin (PLY) plays a significant role in pneumococcal cytotoxicity against alveolar macrophages (AM) and epithelial cells [2-4]. *S. pneumoniae*-triggered replacement of resident alveolar macrophages by exudate macrophages follows remarkable kinetics, thereby pointing to a potent inflammatory monocyte immigration into the alveolar compartment to re-establish AM pool sizes [5-7]. Recent reports demonstrated that *S. pneumoniae*-induced inflammatory exudate macrophage recruitment is critically dependent on the monocyte chemoattractant CCL2 binding to its receptor CCR2, thereby triggering PI3Kγ-dependent downstream signalling cascades to facilitate inflammatory monocyte immigration into the lung [7, 8]. Support for a specific role for CCL2 gradients as a driving force in inflammatory exudate macrophage recruitment during pneumococcal pneumonia came from studies employing mice with a lung-specific over-expression of CCL2 [7]. Relative to wild-type mice, CCL2 transgenic mice exhibited an increased lung exudate mononuclear phagocyte mobilization, together with an improved pathogen elimination and resolution/repair process in response to *S. pneumoniae* challenge [7]. In contrast, blockade of CCR2, the major receptor for CCL2, CCL7, and CCL12, severely impaired the resolution/repair phase both in CCL2 over-expressing and wild-type mice, with fatal consequences for the lung pathogen eradication process [7]. These data strongly supported a central role for the CCL2-CCR2 axis in the process of inflammatory phagocyte recruitment and the accompanying lung resolution/repair phase in response to lung infection with *S. pneumoniae* [3, 7-9]. On the other hand, another recently published report employing a highly virulent, sepsis-eliciting serotype 3 *S.*

pneumoniae strain failed to demonstrate a role for CCL2 in lung protective immunity against pneumococcal infection [10]. In the current study, we reconciled the role of CCL2 in protective immunity to pneumococcal lung infection. We hypothesized that CCL2 chemokine gradients are more protective in lung innate immunity against “non-invasive” *S. pneumoniae* causing focal pneumonia but not bacteraemia [11]. To test this hypothesis, wild-type mice and CCL2 KO mice were infected with a capsular serotype 19 *S. pneumoniae* strain and subsequently analyzed for bacterial loads, bacteraemia and survival as well as lung exudate mononuclear phagocyte recruitment and lung permeability changes.

Materials and Methods

Mice

Wild-type C57BL/6 mice were purchased from Charles River (Sulzfeld, Germany). CCL2 KO mice, which resulted from backcrossing the CCL2 KO onto the C57BL/6 background for more than ten generations, were kindly provided by Dr. Barrett Rollins, Dana Farber Cancer Research Institute, Harvard Medical School, Boston [12]. Both wild-type mice and CCL2 KO mice were kept under conventional conditions with free access to food and water. Mice were used in all experiments at 8-12 wk of age in accordance with the guidelines of our Institutional Animal Care and Use Committee of Hannover School of Medicine, Germany. Animal experiments were approved by our local government authorities.

Culture and quantification of *Streptococcus pneumoniae*

For infection studies, we used a not normally invasive, pneumolysin-producing clinical isolate of capsular group 19 *S. pneumoniae* EF3030. The bacteria were grown in Todd-Hewitt broth (THB) (Difco) supplemented with 0.1% yeast extract to mid-log phase and aliquots were snap-frozen in liquid nitrogen and stored at -80°C until use, as outlined in detail recently [7, 8, 11]. Quantification of *S. pneumoniae* stocks was done by plating serial dilutions on sheep blood agar plates (BD Biosciences, Heidelberg, Germany) and incubation of the plates at 37°C/ 5% CO_2 for 18 h, followed by determination of colony-forming units (CFU).

Infection of mice with *Streptococcus pneumoniae*

CCL2 KO mice and wild-type mice were either mock-infected (50 µl THB) or were infected with *S. pneumoniae* using freshly prepared dilutions of thawed aliquots adjusted to approximately 1.5 x 10^7 CFU/mouse, as recently described [8]. Briefly,

tracheas were exposed by surgical resection, and intratracheal instillation of *S. pneumoniae* was performed under stereomicroscopic control (Leica MS 5, Wetzlar, Germany) using a 26-gauge catheter (Abbocath) inserted into the trachea. After instillation, the neck wound was closed with sterile sutures. In all experiments, mice were kept under SPF conditions with free access to autoclaved food and water and were monitored twice daily for disease symptoms and survival during the entire observation period. For detection of bacteraemia, tail tips were cut and approximately 5-10 µl blood was collected and plated on sheep blood agar plates followed by incubation at 37°C/ 5% CO_2 for 18 h [13].

Determination of bacterial loads in bronchoalveolar lavage and lung parenchymal tissue of CCL2 KO and wild-type mice challenged with *S. pneumoniae*

Bacterial loads in the lungs of *S. pneumoniae*-infected CCL2 KO and wild-type mice were determined in both bronchoalveolar lavage (BAL) fluid and lung tissue homogenates. Briefly, mice were euthanized with an overdose of isoflurane, tracheas of the mice were exposed and cannulated with a shortened 20 G needle that was firmly fixed to the trachea. Subsequently, 300 µl aliquots of ice-cold sterile PBS were instilled and carefully aspirated until a first BAL fluid volume of 1.5 ml was collected. Subsequently, the collection of BAL was continued until an additional BAL fluid volume of 4.5 ml was collected. The 1.5 ml and 4.5 ml BAL fluids (whole lung washes) collected from *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 ten-fold serial dilutions on sheep blood agar plates followed by incubation of the plates at 37°C/5 % CO_2 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 1,400 rpm (4°C, 10 min) and cell

pellets were pooled to determine total numbers of BAL fluid leukocytes. In addition, BAL fluid cytokines were measured in cell-free BAL fluid supernatants of the individual 1.5 ml BAL fluid aliquots. Subsequent to the collection of BAL, lung tissue was homogenized in 2 ml of HBSS without supplements using a tissue homogenizer (IKA, Staufen, Germany), and 10-fold serial dilutions of the lung tissue homogenates were plated on sheep blood agar plates followed by incubation of the plates at 37°C/5% CO_2 for 18 h for CFU determination.

In vivo lung permeability assay

To analyze the lung permeability in mice subsequent to *S. pneumoniae* infection, mice received an intravenous injection of FITC-labeled human albumin (1 mg/mouse in 100 µl saline) (Sigma, Deisenhofen, Germany) 1 h before mice were euthanized by an overdose of isoflurane. Undiluted BAL fluid samples and serum samples (diluted 1/10 in saline) were placed in a 96-well microtiter plate, and fluorescence intensities were measured using a fluorescence spectrometer (FL 800 microplate fluorescence reader) (Biotek, Bad Friedrichshall, Germany) operating at a 488 nm excitation and 525 ± 20 nm emission wavelength. The lung permeability index is defined as the ratio of fluorescence signals of undiluted BAL fluid samples to fluorescence signals of 1/10 diluted serum samples [14].

Immunophenotypic analysis of mononuclear phagocyte subsets in bronchoalveolar lavage

Mononuclear phagocyte subset populations contained in the bronchoalveolar lavage fluid of mock-infected or *S. pneumoniae*-challenged CCL2 KO and wild-type mice were subjected to immunophenotypic analysis of their cell surface antigen expression profiles using a BD FACSCanto flow cytometer equipped with an argon ion laser op-

erating at 488 nm excitation wavelength and a helium neon (HeNe) laser operating at 633 nm wavelength (BD Biosciences, Heidelberg, Germany). Cells pre-incubated with octagam (Octapharma, Langenfeld, Germany) were stained for 15 min at 4°C with various combinations of appropriately diluted fluorochrome-conjugated monoclonal antibodies with specificities for the following cell surface molecules: PE-Cy7-conjugated anti-CD11b, PE-Cy5.5-conjugated anti-CD11c (from BD Biosciences) and APC-conjugated anti-F4/80 (Serotec, Düsseldorf, Germany) [7, 8, 15]. Subsequently, cells were washed in PBS/0.1% BSA/0.02% sodium azide, and cell acquisition was performed on a BD FACSCanto flow cytometer (BD Biosciences). Gating of the respective mononuclear phagocyte subsets was done according to their FSC-A versus SSC-A characteristics and FSC-A versus F4/80-APC fluorescence emission characteristics to exclude debris and contaminating neutrophils from further analysis. Data analysis and careful post-acquisition compensation of spectral overlaps between the various fluorescence channels was performed using BD FACSDiva software (BD Biosciences).

Lung histopathology

Lungs of CCL2 KO and wild-type mice infected with *S. pneumoniae* for different time intervals were inflated *in situ* with PBS-buffered (pH 7.0) formaldehyde solution (4.5 %; ROTH, Karlsruhe, Germany), and then carefully removed and immersed en bloc in formaldehyde solution for at least 24 h at room temperature. Lung tissue samples were then paraffin-embedded and lung sections of 5 µm were stained with Hematoxylin-Eosin (HE) and examined histopathologically using a Zeiss Axiovert 200 M microscope (Zeiss, Göttingen, Germany).

ELISA

Proinflammatory cytokines in BAL fluids of uninfected or *S. pneumoniae*-infected CCL2 KO and wild-type mice were determined using commercially available enzyme-linked immunosorbent assays according to the manufacturer's instructions (ELISA; R&D Systems, Wiesbaden, Germany).

Statistics

All data are given as mean ± SD. Differences between 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 15.0 for Windows software package. Survival curves were compared by log-rank test. Statistically significant differences between groups were assumed when p values were <0.05.

Results

Effect of CCL2 deficiency on survival, bacterial clearance and lung permeability in mice infected with *S. pneumoniae*

We challenged CCL2 deficient mice and wild-type mice with a capsular serogroup 19 strain of *Streptococcus pneumoniae* (EF 3030), which is known to cause focal pneumonia in mice without inducing sepsis [11]. In this study we determined levels of bacteremia, pathogen elimination from the lung, lung permeability, and survival of the mice over a 14-day period following intratracheal inoculation of EF3030. As shown in Fig. 1A, infection of wild-type mice with *S. pneumoniae* EF3030 (~1.5 x 10^7 CFU/mouse) did not cause any mortality during the 14 day observation period, whereas CCL2 KO mice responded with a significant mortality, reaching ~55% by day 10. Importantly, all of the CCL2 KO mice succumbing to pneumococcal challenge developed bacteraemia, whereas none of the wild-type mice developed bacteraemia during lung infection with *S. pneumoniae* (Fig. 1A, B). Analysis of bacterial loads in CCL2 KO as compared to wild-type mice revealed profound differences between groups, with CCL2 KO mice exhibiting significantly increased CFU counts both in BAL fluids and lung tissue samples up until day 7 post-infection (Fig. 1C,D). Notably, a significant increase in lung permeability was observed in the CCL2 KO but not wild-type mice at day 3 post-infection, just when bacteraemia started to develop in the mutant mice (Fig. 1 E).

Histological analysis of lung tissue sections collected from CCL2 KO mice and wild-type mice revealed a normal lung architecture in mice under baseline conditions (Fig. 2, mock-infection, 0 d). Upon *S. pneumoniae* infection, the lungs of CCL2 KO mice but not wild-type mice demonstrated strongly increased leukocytic infiltrates, mainly consisting of apoptotic/necrotic neutrophils, along with alveolar edema forma-

tion consistent with lobar pneumonia, starting by day 3 and peaking by day 7 post-infection (Fig. 2).

Proinflammatory cytokine responses in *Streptococcus pneumoniae* infected CCL2 KO mice and wild-type mice

We next analyzed proinflammatory mediator profiles in BAL fluids of CCL2 KO and wild-type mice infected with *S. pneumoniae*. As shown in Fig. 3, proinflammatory cytokines TNF-α (A) and IL-6 (B) rapidly increased in BAL fluids of both CCL2 KO mice and wild-type mice by day 1 post-infection, and rapidly declined thereafter towards baseline levels in the wild-type mice, whereas CCL2 KO mice demonstrated sustained elevated levels of TNF-α and IL-6 until day 7-14 post-infection. Similar differences in cytokine responses between groups were also noted for neutrophil chemoattractants MIP-2 and KC, which rapidly declined in the wild-type mice but remained elevated until day 7 post-infection in the KO mice (Fig. 3C, D). As anticipated, monocyte chemoattractant CCL2 was only detectable in the wild-type mice, where it rapidly peaked by day 1 post-infection and declined towards baseline levels by day 7 of infection with *S. pneumoniae*. Levels of monocyte chemoattractants CCL7 and CCL12 were more pronounced in BAL fluids of CCL2 KO mice as compared to wild-type mice, with peak levels observed on days 3 and 7 post-challenge with *S. pneumoniae*.

Effect of CCL2 deficiency on lung exudate macrophage recruitment and alveolar macrophage repopulation subsequent to infection with *S. pneumoniae*

Lung exudate macrophages are critically important in the removal of consolidated infiltrates during the resolution/repair phase of bacterial lung infections [6-8, 16]. Thus, a rapidly executed lung macrophage recruitment subsequent to pulmonary in-

fection with *S. pneumoniae* is important for a rapid reconstitution of lung tissue integrity and function [6-8]. Because CCL2 KO mice demonstrated a severely impaired lung resolution/repair phase particularly on day 7 post-infection, we analyzed inflammatory lung exudate macrophage mobilization in these mice upon challenge with *S. pneumoniae*. FACS analysis of BAL fluid cellular constituents of wild-type mice and CCL2 KO mice revealed similar numbers of resident alveolar macrophages (P5 in Fig. 4E,H) and a detectable, but very low lung exudate macrophage recruitment in either experimental group under baseline (mock-infection) conditions (P6 in Fig. 4E, H). Notably, wild-type mice infected with *S. pneumoniae* demonstrated a well-coordinated lung inflammatory exudate macrophage recruitment (P6 in Fig. 4F, G, and Fig. 4L) and alveolar macrophage repopulation upon infection with *S. pneumoniae* with peak macrophage numbers noted between days 3 and 7 post-infection (P5 in Fig. 4F, G, and Fig. 4K). In contrast, CCL2 KO mice responded with a significantly impaired resident alveolar macrophage repopulation upon infection with *S. pneumoniae* (P5 in Fig. 4IJ, and Fig. 4K) up until day 7 post-infection that was accompanied by an equally impaired inflammatory lung exudate macrophage recruitment observed from day 1 up until day 7 post-infection, i.e. just when formation of lung consolidated infiltrates in mutant mice reached its maximum (P6 in Fig. 4I, J, and Fig. 4L). These data support the conclusion that the profound defect in inflammatory lung exudate macrophage recruitment in the CCL2 KO mice was most likely responsible for the observed impaired reconstitution of lung homeostasis subsequent to infection with *S. pneumoniae.* Moreover, in line with increased levels of neutrophil chemoattractants MIP-2 and KC in their BAL fluids, mutant mice recruited significantly more neutrophils into their lungs upon infection with *S. pneumoniae* compared to wild-type mice (Fig. 4M), thereby, in cohort with the reduced macrophage recruitment, further aggravating the formation of consolidated infiltrates and lung tissue destruction.

Discussion

In the current study, we determined whether a lack of CCL2-dependent lung exudate macrophage recruitment would enable otherwise non-invasive group 19 *S. pneumoniae* to escape local immune surveillance and cause septic disease progression in mice. CCL2 deficient mice demonstrated a rapidly depleted resident alveolar macrophage pool and sustained reduction in lung exudate macrophage recruitment upon infection with capsular group 19 *S. pneumoniae* EF3030. At the same time, they developed bacteraemia and >50% mortality by day 10 post-infection, which was not observed in *S. pneumoniae*-infected wild-type mice. Interestingly, increased neutrophil recruitment observed in the infected CCL2 KO mice compared to wild-type mice could not compensate for the loss of lung exudate macrophage recruitment in the mutant mice. These data refine the role of the monocyte chemoattractant CCL2 in lung protective immunity against inhaled bacterial pathogens by demonstrating that chemokine-driven lung exudate macrophage recruitment serves as a “barrier” to inhibit escape of otherwise non-invasive *S. pneumoniae* from local lung compartments to cause sepsis in mice.

Pathogenicity profiles vary among different *S. pneumoniae* serotypes, allowing the bacteria to either cause relatively uncomplicated lobar pneumonia or pneumonia followed by rapidly developing bacteraemia and invasive pneumococcal disease, thereby leading to high morbidity and mortality [17]. Pneumococcal infections of the lung trigger the development of consolidated infiltrates, which are largely composed of apoptotic and necrotic neutrophils, fibrin-rich alveolar exudate fluid and incorporated bacteria, and which contribute to impaired gas exchange and respiratory distress. Therefore, recruitment of exudate macrophages to sites of infection is an im-

portant process for the resolution of consolidated infiltrates to regain lung tissue integrity and function subsequent to bacterial challenge [7, 8].

Recent studies from our lab showed that mice over-expressing the monocyte chemoattractant CCL2 in their lungs exhibited an improved protective immunity against *S. pneumoniae* challenge, but with the consequence of bronchiolitis obliterans formation in the later phase of disease [7]. Importantly, that study addressed the question whether lung protective immunity against inhaled bacterial pathogens can be “optimized” with respect to pathogen elimination and resolution/repair mechanisms. In addition, the employed model system of lung-specific CCL2 overexpression was characterized by pre-expanded pools of mononuclear phagocyte subsets in the lung before the pathogen (a clinical isolate of serotype 19 *S. pneumoniae* known to primarily cause focal pneumonia in mice [11]) was delivered into the lungs. Therefore, one may consider that CCL2 might have been effective only because the CCL2 chemokine gradient and accompanying exudate macrophage recruitment was pre-established in an infection model, where the employed pathogen is known to usually not cause invasive disease. In this regard, a recent study by Dessing et al. reported that CCL2 did not contribute to protective immunity when employing a capsular serotype 3 *S. pneumoniae* strain, which is known to rapidly cause bacteraemia and sepsis in mice [10].

However, when considering the kinetics by which monocyte chemokine gradients are established and the subsequently elicited macrophage recruitment to sites of infection is executed, it appears that lung protective immunity is most efficient under those conditions where the host is able to mount the appropriate leukocytic responses before the pathogen overcomes barrier cells of the lung (i.e., alveolar epithelial and capillary endothelial cells) to cause bacteraemia and sepsis. As such, we believe that definition of a pneumococcal strain as ‘non-invasive’ not only de-

pends on its intrinsic pathogenicity profile, but also depends, at least in part, on the host's ability to rapidly mount an appropriate innate immune response to impair bacterial outgrowth, as observed here with "non-invasive" serotype 19 *S. pneumoniae* EF3030 that caused bacteraemia and mortality in mutant but not wild-type mice. Also, a phenotypic comparison of pneumococcal infection models should always take into account that both capsular and non-capsular virulence factors contribute to invasiveness of a given strain. From a clinical perspective, CCL2 deficiency appears to resemble immunosuppression, where the host looses its ability to control otherwise harmless infections. Pharmacological approaches aimed at inhibiting inflammatory monocyte recruitment to sites of inflammation, similar to pharmacological intervention strategies to target CCL2 receptor downstream signalling cascades [8], may be expected to alter the susceptibility of the lung for inhaled pathogens.

In a recent study by Knapp and colleagues, it was shown that depletion of alveolar macrophages by liposomal clodronate led to a highly increased proinflammatory cytokine release in mice upon infection with *S. pneumoniae*, which was associated with a poor outcome [16]. These observations gave rise to the concept that macrophages are involved in the regulation of proinflammatory cytokine production in response to *S. pneumoniae* infection. In the current study, deletion of CCL2 led to decreased numbers of resident alveolar and particularly exudate macrophages until day 7 post-infection, with a sustained cytokine release in the KO mice but not wild-type mice over time. These data extend previous reports and suggest that possibly both resident alveolar *and* recruited exudate macrophages, which are phenotypically different phagocyte populations (Fig. 4, and [6]), are involved in the regulation of proinflammatory cytokine responses during pneumococcal lung infection.

The current study provides novel insights regarding the role of CCL2 chemokine gradients and the accompanying macrophage recruitment in the lung host

defense against “non-invasive” *S. pneumoniae.* These data may have clinical relevance in view of emerging anti-inflammatory strategies to attenuate inflammatory neutrophil and/or exudate macrophage recruitment in chronic diseases [18, 19], which might exert unwanted remote effects on lung protective immunity against inhaled bacterial pathogens.

Footnote page

[1]**Conflict of Interest declaration:** Christine Winter: No conflict of interest; Regina Maus: No conflict of interest; David E. Briles: No conflict of interest; James C. Paton: No conflict of interest; Tobias Welte: No conflict of interest; Ulrich A. Maus: No conflict of interest.

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Figure Legends

Figure 1. Survival, bacteraemia, and pathogen elimination in CCL2 deficient mice infected with *S. pneumoniae.* Wild-type mice and CCL2 KO mice were infected with ~1,5x10^7 CFU of serotype 19 *S. pneumoniae* EF3030 and survival (A) and cumulative bacteraemia (B) were determined up until 14 days post-infection (wild-type, n=10 mice/group; CCL2 KO, n=11 mice/group). (C,D) Bacterial loads in BAL fluids (C) and lung tissue homogenates (D) of wild-type mice (white bars) or CCL2 KO mice (black bars) for various time intervals, as indicated. (E) One hour before sacrifice, *S. pneumoniae*-infected wild-type mice or CCL2 KO mice received intravenous injections of FITC-labeled human albumin (1mg/mouse) for fluorimetric analysis of lung permeability subsequent to *S. pneumoniae* infection. In (C-E), values are shown as mean±SD of n=5-10 mice per treatment group and time-point. * indicates significant difference ($p<0.05$) compared to wild-type mice. ++++ indicates significant difference ($p<0.0001$) to the respective CFU value at day 1 post-infection.

Figure 2. Histopathology of CCL2 KO mice and wild-type mice infected with *S. pneumoniae.* Wild-type mice and CCL2 KO mice were infected with ~1,5x10^7 CFU of serotype 19 *S. pneumoniae* EF3030. At the indicated time points, mice were euthanized and lungs were removed for assessment of histopatholgical manifestations. The shown photomicrographs of paraffin-embedded lung tissue sections (5 µm) were taken at a x10 original magnification and are representative of n=5 mice per group and time point.

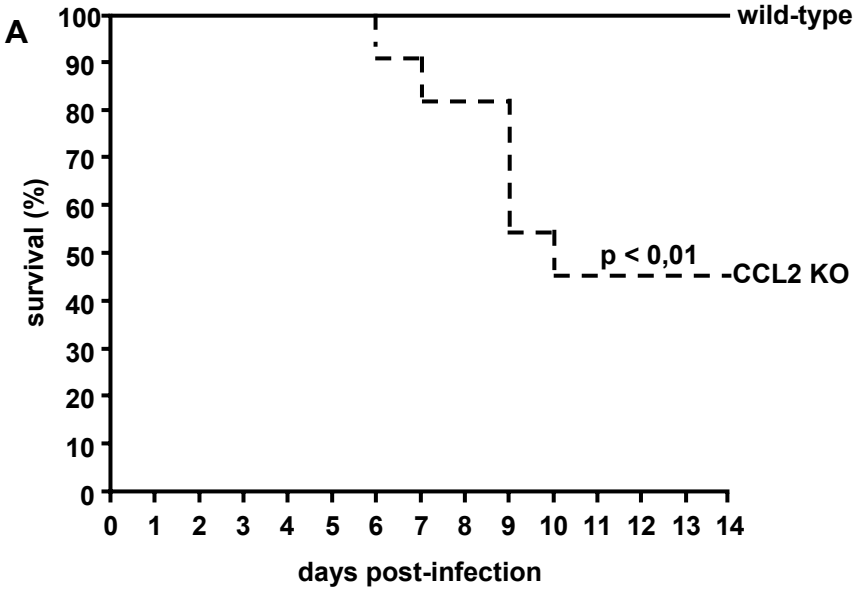
Figure 3. Proinflammatory cytokine responses in *Streptococcus pneumoniae* infected CCL2 KO mice compared to wild-type mice. Wild-type mice (white bars) and CCL2 KO mice (black bars) were either mock-infected (50 µl THB, 0 d time

points) or were infected with serotype 19 *S. pneumoniae* EF3030 (~$1.5x10^7$ CFU/mouse). At the indicated time points, mice were euthanized and subjected to bronchoalveolar lavage for the determination of cytokine profiles, as indicated. Values are shown as mean ± SD of n = 5-10 mice per treatment group and time-point. *, significant difference ($p<0.05$; ****, $p < 0.0001$) compared to wild-type. +, significant difference ($p<0.05$; ++++, $p < 0.0001$) compared to the respective control values (d 0).

Figure 4. Effect of CCL2 deficiency on lung exudate macrophage recruitment and alveolar macrophage repopulation subsequent to infection with *S. pneumoniae.* Wild-type mice and CCL2 KO mice were either mock-infected (A-D, E,H, and 0 d time points in K-M) or were infected with *S. pneumoniae* (F-J, K-M), as described in the legend to Fig. 3. Subsequently, mice were euthanized at the indicated time points and BAL fluid cellular constituents were subjected to FACS analysis for characterization of numbers of resident alveolar macrophages or exudate macrophage recruitment profiles. BAL fluid cells of mice from either treatment group were gated according to their FSC-A versus SSC-A characteristics to exclude cellular debris (P1 and P3 in A, B). Subsequently, F4/80-positive mononuclear phagocytes (P2 (wild-type) and P4 (CCL2 KO) in C,D) were analyzed for their CD11c versus CD11b cell surface antigen expression to discriminate between resident (P5 in E,H, and F-J) and exudate macrophages (P6 in E,H, and F-J) under baseline, mock-infection conditions or in response to *S. pneumoniae* infections, as indicated. Filled histograms represent isotype-stained negative controls, and overlay histograms represent F4/80 specific cell surface Ag expression. The cumulative data from the above described FACS analyses are given in K and L and represent mean ± SD values of n=5-10 mice per treatment group and time-point. * indicates significant difference ($p<0.05$; **,

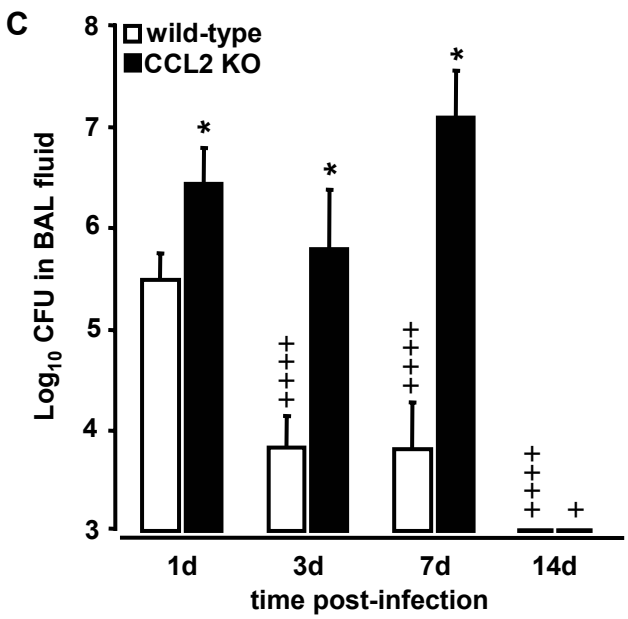
p<0.01; ***, p<0.001) compared to wild-type. + indicates significant difference (p<0.05; ++, p<0.01; +++, p<0.001; ++++, p < 0.0001) compared to the respective mock-infected control values (d 0).

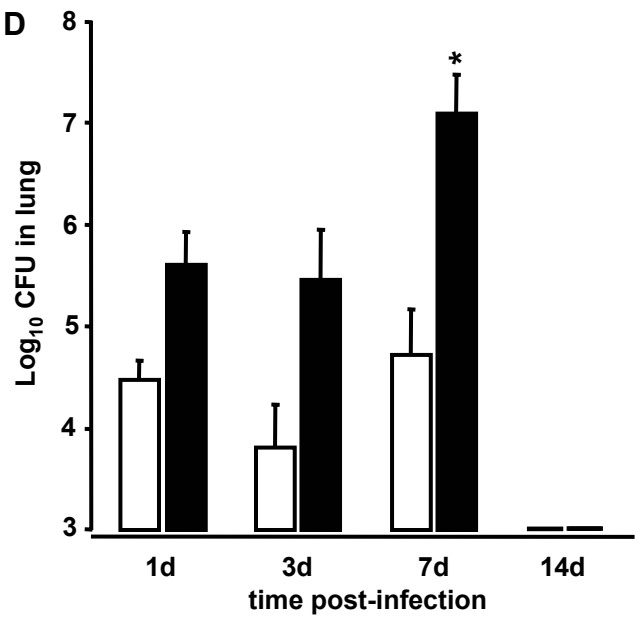
Figure 1



B

days p.i.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
wt bacteraemia	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
KO bacteraemia	0/11	0/11	2/11	2/11	3/11	3/11	3/11	3/11	4/11	5/11	5/11	5/11	5/11	5/11





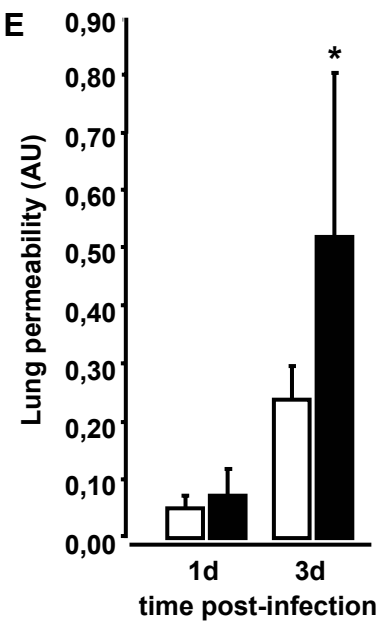


Figure 2

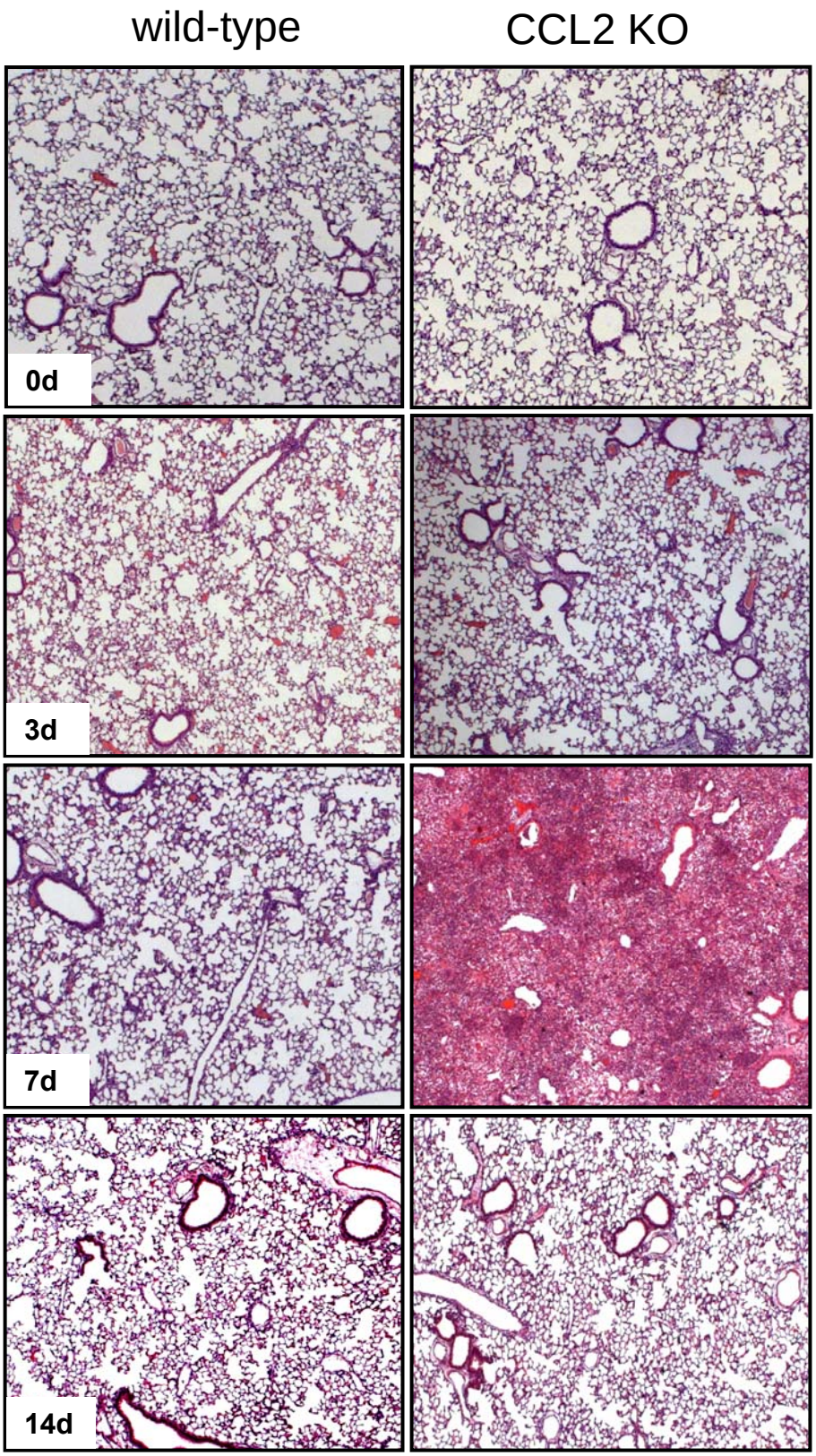


Figure 3

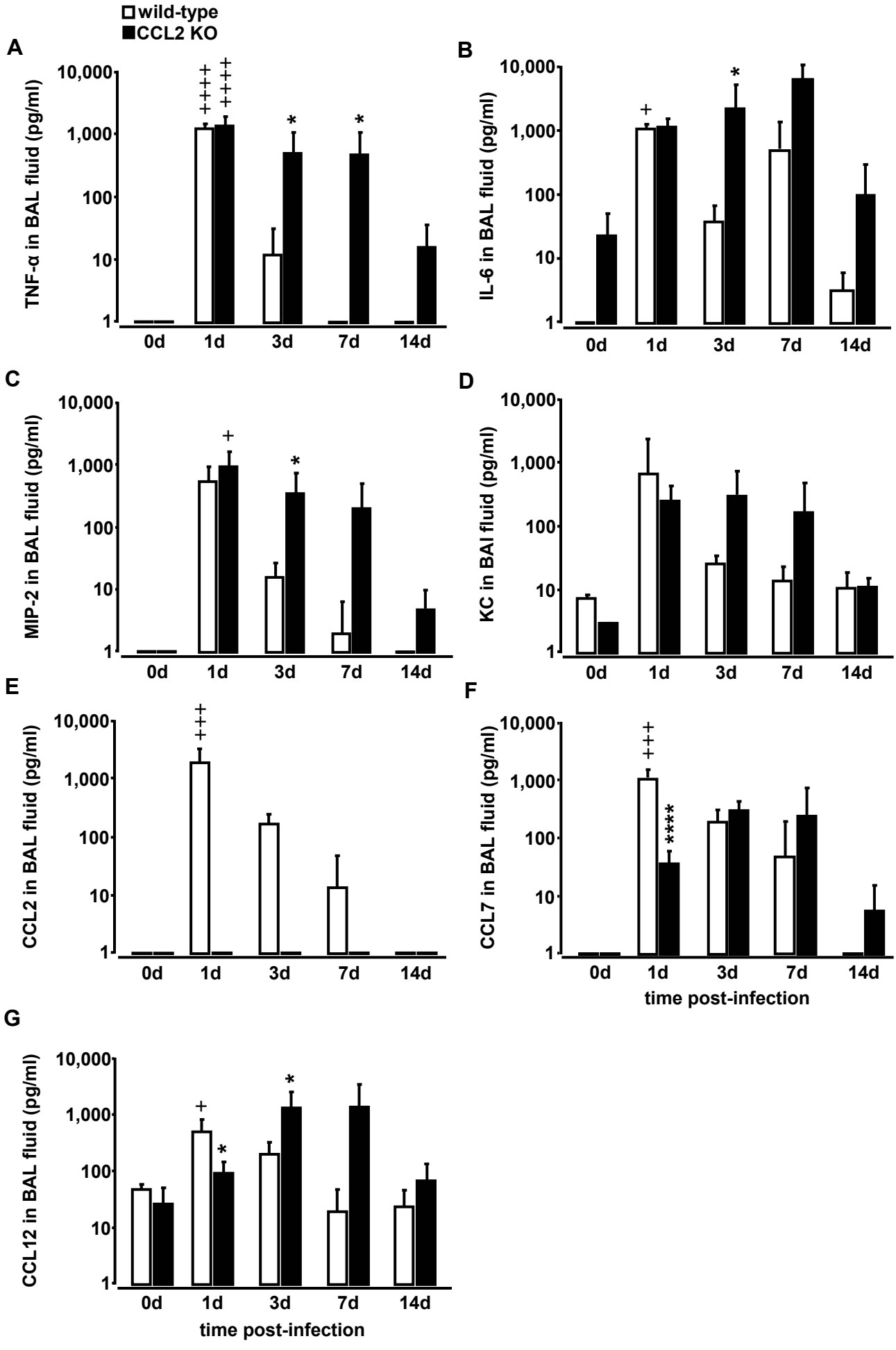
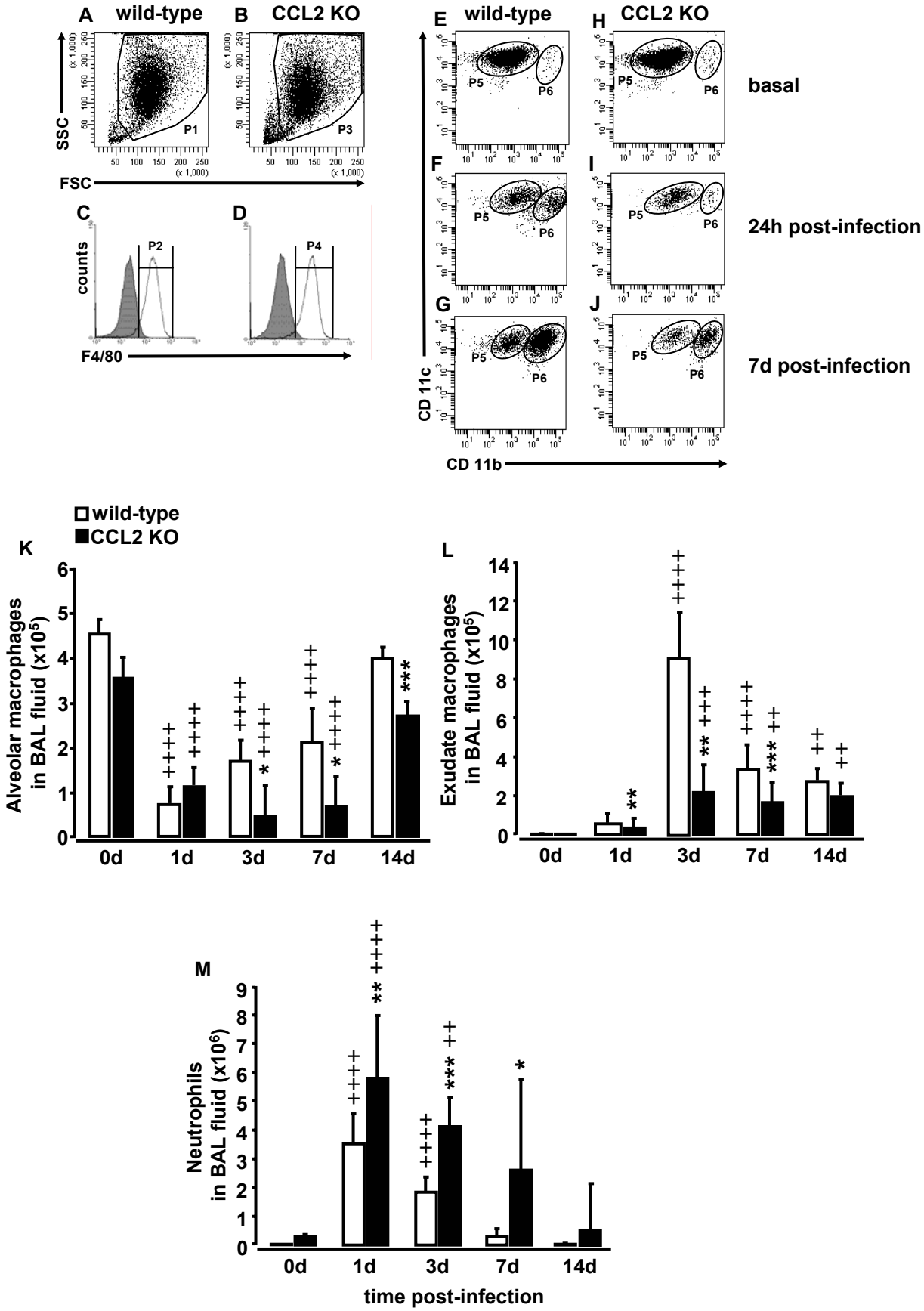

wild-type
CCL2 KO
A
TNF-α in BAL fluid (pg/ml)
B
IL-6 in BAL fluid (pg/ml)
C
MIP-2 in BAL fluid (pg/ml)
D
KC in BAl fluid (pg/ml)
E
CCL2 in BAL fluid (pg/ml)
F
CCL7 in BAL fluid (pg/ml)
G
CCL12 in BAL fluid (pg/ml)
10,000
1,000
100
10
1
0d
1d
3d
7d
14d
time post-infection

Figure 4


A wild-type
B CCL2 KO
SSC
FSC
P1
P3
C
D
counts
F4/80
P2
P4
E wild-type
H CCL2 KO
basal
F
I
24h post-infection
G
J
7d post-infection
CD 11c
CD 11b
P5
P6
wild-type
CCL2 KO
K
Alveolar macrophages in BAL fluid (x10⁵)
L
Exudate macrophages in BAL fluid (x10⁵)
M
Neutrophils in BAL fluid (x10⁶)
0d
1d
3d
7d
14d
time post-infection