Lung epithelial apoptosis in influenza virus pneumonia: the role of macrophage-expressed TNF-related apoptosis-inducing ligand

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Mononuclear phagocytes have been attributed a crucial role in the host defense toward influenza virus (IV), but their contribution to influenza-induced lung failure is incompletely understood. We demonstrate for the first time that lung-recruited “exudate” macrophages significantly contribute to alveolar epithelial cell (AEC) apoptosis by the release of tumor necrosis factor–related apoptosis–inducing ligand (TRAIL) in a murine model of influenza-induced pneumonia. Using CC-chemokine receptor 2–deficient (CCR2−/−) mice characterized by defective inflammatory macrophage recruitment, and blocking anti-CCR2 antibodies, we show that exudate macrophage accumulation in the lungs of influenza-infected mice is associated with pronounced AEC apoptosis and increased lung leakage and mortality. Among several proapoptotic mediators analyzed, TRAIL messenger RNA was found to be markedly up-regulated in alveolar exudate macrophages as compared with peripheral blood monocytes. Moreover, among the different alveolar-recruited leukocyte subsets, TRAIL protein was predominantly expressed on macrophages. Finally, abrogation of TRAIL signaling in exudate macrophages resulted in significantly reduced AEC apoptosis, attenuated lung leakage, and increased survival upon IV infection. Collectively, these findings demonstrate a key role for exudate macrophages in the induction of alveolar leakage and mortality in IV pneumonia. Epithelial cell apoptosis induced by TRAIL–expressing macrophages is identified as a major underlying mechanism.

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These “exudate” macrophages acquire a lung resident macrophage phenotype and finally replenish the alveolar macrophage pool during the time course of infection (7).

Besides their essential host defense functions, mononuclear phagocytes have been proposed to contribute to an imbalanced detrimental immune response during IV pneumonia (8, 9) and have been implicated in alveolar epithelial damage. Human IV pneumonia, which is characterized by rapid progression to lung failure and poor outcome, has gained in importance during the recent outbreaks in Southeast Asia. Infection with highly pathogenic IV causes significant tissue damage to the lungs with acute alveolitis followed by massive pulmonary edema and hemorrhage and extensive destruction of the respiratory epithelium (10–12). However, the distinct molecular steps of macrophage–epithelial interaction during IV-induced acute lung injury remain elusive.

Several authors suggest AEC apoptosis to be an underlying mechanism of alveolar damage in murine and human models of adult respiratory distress syndrome (13–15). Death receptors and their ligands play an important role in the orchestration of innate and adaptive immune responses (16–18). TNF-related apoptosis-inducing ligand (TRAIL) is a membrane protein belonging to the TNF superfamily. Among the members of this family, TRAIL exhibits the highest homology to Fas ligand (FasL), a well known inducer of programmed cell death (18). Being expressed mainly on T cells, NK cells, and mononuclear phagocyte subsets, murine TRAIL exerts its proapoptotic signals via binding to DR5 (death receptor 5) (19) and displays potent antitumor activity (20, 21). Recently, an antiviral function in experimental murine IV infection has been suggested (18). However, the contribution of TRAIL to alveolar epithelial apoptosis and lung barrier dysfunction during lethal IV infection has not been elucidated yet.

In the present study, in a murine model of IV-induced acute lung injury, we demonstrate for the first time that exudate macrophages recruited via the CCL2–CCR2 axis largely contribute to AEC apoptosis involving the expression of TRAIL. Both blockade of the specific chemokine-receptor axis and abrogation of macrophage TRAIL signaling by anti-TRAIL mAb treatment or use of adoptively transferred mice recruiting TRAIL-deficient exudate macrophages significantly reduced alveolar epithelial apoptosis and lung leakage in infected mice, resulting in increased survival during otherwise fatal IV pneumonia.

RESULTS
Genetic deletion of CCR2 reduces mortality, morbidity, and alveolar barrier dysfunction during PR/8 infection

Previous reports suggest that the chemokine receptor CCR2 is critically involved in host immune responses to lung infection (4, 22–24). To investigate the influence of CCR2 on the course of IV pneumonia in mice, C57BL/6 WT and CCR2−/− mice were intratracheally inoculated with a lethal dose of the mouse-adapted IV strain PR/8, and survival and body weight were determined during the 21 d post infection (pi). As shown in Fig. 1 A, only 17.3% of CCR2-deficient mice succumbed to PR/8 infection as compared with 78.4% of infected WT mice (P < 0.005 on days 14–21 pi). Likewise, body weight loss was significantly less in CCR2−/− mice on days 8, 9, and 11 pi. To evaluate whether the observed differences in morbidity and mortality during PR/8 infection were associated with increased severity of lung injury in WT mice as compared with CCR2−/− mice, alveolar barrier function was assessed during a time course of 21 d after PR/8 infection in the two treatment groups. Indeed, alveolar leakage was significantly reduced in CCR2-deficient mice on day 7 pi (1.25 ± 0.39 vs. 0.54 ± 0.37 arbitrary units; Fig. 1 B), indicating that CCR2−/− mice develop less alveolar barrier damage upon PR/8 infection than WT mice.

During IV pneumonia, alveolar epithelial injury may be caused by direct cytopathic effects of IV replicating primarily in epithelial cells. Therefore, we evaluated whether the observed differences in lung barrier damage between WT and CCR2−/− mice were linked to different viral replication efficiencies in the lung tissue of the two mouse strains. Analyses of viral replication in lung homogenates from PR/8-infected WT compared with CCR2−/− mice revealed no significant differences in peak viral titers at days 2, 3, and 5 pi and even slightly elevated virus titers in CCR2−/− mice during the later stages of infection (1.05 ± 1.33 × 10^4 vs. 6.20 ± 3.90 × 10^4 foci-forming units/lung on day 11 pi; Fig. 1 C). These
counts and cell differentials were determined at various time points pi. No significant differences in total BALF cell counts were detectable between the two treatment groups (Fig. 2 A). Peak alveolar neutrophil accumulation was delayed in CCR2-deficient mice and slightly exceeded values reached in WT mice on days 7 and 8 pi. Lymphocyte recruitment was virtually identical in both treatment groups. Total numbers of resident alveolar macrophages in BALF were lower in CCR2-deficient mice on days 5–9 pi but reached comparable levels during the later stages of infection. However, alveolar mononuclear phagocyte recruitment was found to be strongly reduced in CCR2-deficient mice, most prominently at day 8.

CCR2 deficiency selectively affects alveolar mononuclear phagocyte recruitment during IV pneumonia
To evaluate whether the amount and composition of alveolar leukocyte infiltration differ in PR/8-infected WT and CCR2−/− mice, total bronchoalveolar lavage (BAL) fluid (BALF) cell

Figure 2. Alveolar macrophage recruitment in PR/8-infected mice is CCR2 dependent. (A and B) PR/8-infected WT (filled bars) or CCR2−/− (empty bars) mice were subjected to BAL, followed by quantification of total leukocyte BALF numbers (A) and BAL leukocyte subpopulations (B) from Pappenheim-stained cytocentrifuge preparations. (C) Quantification of exudate mononuclear phagocytes calculated from differential counts of BAL leukocytes from infected WT mice treated with isotype IgG or anti-CCR2 mAb (day 3 pi, left). Alveolar leakage of isotype- or anti-CCR2–treated PR/8-infected mice (day 7 pi, right). (D) FACS analysis of alveolar mononuclear phagocyte subpopulations in BALF from infected WT versus CCR2−/− mice or from WT mice treated with isotype IgG or anti-CCR2 mAb was performed by three-hierarchy gating on day 8 pi. CD45+ cells (population 1 [P1]) were gated according to their GR1 and F4/80 expression. GR1+ F4/80− cells represent alveolar mononuclear phagocytes (population 2 [P2]). Subgate analysis of population 2 revealed a CD11cint (population 3 [P3]), exudate macrophages) and a CD11c+ MHCII+ (population 4 [P4], resident AM) subpopulation as well as a CD11c+ MHCII+ subpopulation (population 5 [P5]) representing alveolar DCs. (E) Flow cytometric quantification of CD11c+ MHCII+ DCs and lymphocyte subpopulations from BALF leukocytes of PR/8-infected WT (filled bars) versus CCR2−/− (empty bars) mice using the gating characteristics described in D for DCs. For analysis of lymphocyte subpopulations, CD45+ cells were subgated on NK cells (SSC−NK1.1+), CD4 T cells (SSC−CD4+), and CD8 T cells (SSC−CD8+). Data in E are given as total cells in BAL calculated from the respective percentage of CD45+ cells. All bar graphs represent the means ± SD from five experiments. *, P < 0.05; **, P < 0.005. AM, alveolar macrophages; ex, exudate; mn, mononuclear; iso, isotype IgG.
pi (1.27 ± 0.35 × 10^6 vs. 0.3 ± 0.21 × 10^6; Fig. 2 B). Correspondingly, exudate mononuclear phagocyte numbers were significantly decreased in PR/8-infected WT mice pretreated with an anti-CCR2 mAb by day 8 pi (Fig. 2 C, left), which is associated with reduced alveolar leakage, as compared with isotype IgG-treated mice (Fig. 2 C, right), suggesting a key role of alveolar recruited mononuclear phagocytes in IV-induced lung barrier breakdown.

To further dissect the alveolar mononuclear phagocyte subset composition, BALF cells gained from PR/8-infected mice of the various treatment groups were subjected to FACS analysis for surface marker expression at day 8 pi. Analysis of CD45+ BALF cells (population 1; Fig. 2 D, first column) for GR1 and F4/80 expression revealed three distinct cell populations: Gr1^high^F4/80^-^ neutrophils, Gr1^-^F4/80^+-^ lymphocytes, and Gr1^int^F4/80^+-^ mononuclear phagocytes (population 2; Fig. 2 D, second column), which were composed of a CD11c^int^ (population 3, henceforth termed exudate macrophages) and

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Figure 3. Alveolar barrier dysfunction is associated with CCR2 expression on circulating leukocytes during PR/8 infection. (A) Three different groups of PR/8-infected BM chimeric mice (WT recipient mice with transplantation of 100% WT BM cells; WT recipient mice with transplantation of mixed 50% WT/50% CCR2^−/−^ BM cells; and WT recipient mice with transplantation of 100% CCR2^−/−^ BM cells) were subjected to BAL at day 8 pi. Exudate macrophage numbers were calculated from BALF leukocyte numbers and differential counts of Pappenheim-stained cytocentrifuge preparations. (B) FACS analysis of BALF cells from PR/8-infected chimeric mice of each transplantation group was performed as described in Fig. 2 D, and representative dot plots from three independent experiments are shown. Note that population 3 (P3; exudate macrophages), population 4 (P4; resident alveolar macrophages), and population 5 (P5; alveolar DCs) are subgates of population 2 [P2]. (C) Alveolar leakage from PR/8-infected chimeric mice at day 7 pi. All bar graphs represent the means ± SD of four to eight mice per group from three independent experiments. ***, P < 0.005.

Figure 4. AEC apoptosis is reduced in PR/8-infected CCR2^−/−^ mice compared with WT mice. (A) WT or CCR2^−/−^ mice were mock or PR/8 infected and TUNEL assay was performed on cryosections from lavaged lungs at day 7 pi. Nuclei of apoptotic cells appear in brown. Arrows, apoptotic intraalveolar leukocytes; arrowheads, apoptotic AEC. Bars, 100 μm. (B) Flow cytometric quantification of apoptotic AEC. Lungs from mock- or PR/8-infected WT or CCR2^−/−^ mice were digested on day 7 pi and analyzed for annexin V binding. Representative dot plots show expression of the AEC type I marker T1α and annexin V staining of viable (propidium iodide negative) CD45^-^ cells. Apoptotic cells are mainly AEC type I (left). Quantification of FACS data as obtained in B from PR/8-infected mice (right). The bar graph represents the annexin V^+^ proportion of CD45^-^ T1α^+^ cells. (C) Quantification of FACS analysis of the NP^+^ proportion of CD45^-^ T1α^+^ cells from lung homogenates in the time course after PR/8 infection. Values are means ± SD of three to four mice per group from three independent experiments. *, P < 0.05; ***, P < 0.005. NP, IV nucleoprotein; filled bars, WT; empty bars, CCR2^−/−^.
a CD11c<sup>high</sup> (population 4) subpopulation, representing resident alveolar macrophages according to previous reports (2, 3). Exudate macrophages (population 3) accumulated to a much lesser extent in CCR2<sup>−/−</sup> mice or in mice pretreated with anti-CCR2 antibody as compared with WT mice or isotype IgG-treated mice, respectively (Fig. 2 D, third column). Alveolar mononuclear phagocytes (population 2) contained a low proportion of DCs (CD11c<sup>+</sup>MHCII<sup>high</sup> [population 5 (P5)]; Fig. 2 D, fourth column). Alveolar DC recruitment was delayed in PR/8-infected CCR2<sup>−/−</sup> mice but reached peak values comparable to those of WT mice by day 8 pi (Fig. 2 E, top left). Flow cytometric analysis of alveolar lymphocyte subsets revealed no striking differences in the recruitment of CD4<sup>+</sup> T cells or CD8<sup>+</sup> T cells between WT and CCR2-deficient mice at the given time points, whereas alveolar NK cell accumulation peaked earlier at day 5 pi in CCR2<sup>−/−</sup> mice (Fig. 2 E). Analysis of interstitial exudate macrophage accumulation in PR/8-infected lungs revealed faster recruitment kinetics than in the alveolar compartment but was likewise significantly reduced in mice lacking CCR2 (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20080201/D1C1). Altogether, our data demonstrate that recruitment of GR<sup>1</sup> int/F4/80<sup>+</sup>CD11c<sup>+</sup>MHCII<sup>high</sup> exudate macrophages into the lungs during PR/8 pneumonia was severely impaired in CCR2-deficient mice or WT animals treated with function blocking anti-CCR2 antibodies.

**CCR2 expression on circulating leukocytes, but not on lung resident cells, is associated with alveolar barrier dysfunction during PR/8 infection**

To distinguish whether CCR2 expressed on resident lung cells, such as resident alveolar macrophages or AEC, or CCR2 present on circulating leukocytes accounted for the increased IV-induced alveolar leakage in WT versus CCR2<sup>−/−</sup> mice, we made use of a BM chimeraic mouse model. We established three different transplantation groups: (a) WT mice with transplantation of 100% WT BM cells; (b) WT mice with transplantation of a mixture of WT and CCR2<sup>−/−</sup> BM cells (50% WT/50% CCR2<sup>−/−</sup>); and (c) WT mice with transplantation of 100% CCR2<sup>−/−</sup> BM cells. CCR2 expression on F4/80<sup>+</sup> peripheral blood monocytes was analyzed 14 d after transplantation by flow cytometry and corresponded to the respective proportion of transplanted WT or CCR2<sup>−/−</sup> BM cells in the different transplantation groups, whereas resident alveolar macrophages homogeneously displayed a WT phenotype as previously outlined in detail (25) (unpublished data). Exudate macrophage accumulation on day 8 after PR/8 infection in transplanted mice was dependent on the proportion of peripheral blood monocytes with intact CCR2 expression, with mice after transplantation of WT BM recruiting 0.85 ± 0.27 × 10<sup>6</sup>, mice after transplantation of mixed BM recruiting 0.57 ± 0.29 × 10<sup>6</sup>, and mice after transplantation of CCR2<sup>−/−</sup> BM recruiting a total of 0.24 ± 0.09 × 10<sup>6</sup> exudate macrophages into the alveolar compartment (Fig. 3, A and B). Notably, alveolar leakage at day 7 pi was significantly reduced in mice after transplantation of CCR2<sup>−/−</sup> BM compared with mice after transplantation of 100% WT BM (Fig. 3 C). These results clearly demonstrate that CCR2 expressed on circulating blood monocytes is critically involved in mononuclear phagocyte extravasation into the alveolar compartment of the lung, thereby contributing to alveolar barrier dysfunction during IV pneumonia.

**CCR2-dependent alveolar exudate macrophage accumulation is associated with increased AEC apoptosis**

Given that the CCR2-dependent accumulation of exudate macrophages in IV pneumonia contributed to the loss of alveolar barrier integrity, we hypothesized that this recruited mononuclear cell population might promote barrier dysfunction by inducing AEC apoptosis. Therefore, cryosections from lavaged lungs of mock- or PR/8-infected WT or CCR2-deficient mice were subjected to TUNEL assay. As shown in Fig. 4 A, the number of apoptotic alveolar cells was strikingly less in PR/8-infected CCR2<sup>−/−</sup> mice as compared with WT mice, and virtually undetectable in mock-infected mice.
mice. For apoptosis quantification of AEC type I representing the major component of the alveolar surface, lung homogenates of the respective treatment groups were subjected to flow cytometry, and CD45<sup>+</sup> cells were analyzed for annexin V binding and expression of the AEC type I marker T1α. Representative dot plots in Fig. 4 B (left) demonstrate a significantly larger proportion of type I AEC to undergo apoptosis in lung homogenates of PR/8-infected WT mice than in PR/8-infected CCR2-deficient mice at day 7 pi. Flow cytometric quantification during the time course of infection revealed a sustained increase of type I AEC apoptosis in WT compared with CCR2<sup>−/−</sup> mice throughout days 7–11 pi corresponding to the kinetics of alveolar exudate macrophage accumulation (Fig. 4 B, right). The proportions of infected (IV nucleoprotein<sup>+</sup>) type I AEC were virtually identical in both WT and CCR2-deficient mice, with direct viral cytopathic effects most likely accounting for the observed type I AEC apoptosis rate that was still present in CCR2<sup>−/−</sup> mice (Fig. 4, B and C). No differences in CD45<sup>−</sup>CD31<sup>+</sup> lung endothelial cell apoptosis could be detected during days 5–14 in PR/8-infected WT compared with CCR2<sup>−/−</sup> mice (unpublished data).

Figure 6. TRAIL is expressed on the cell surface of alveolar exudate macrophages. (A) WT or CCR2<sup>−/−</sup> mice were mock or PR/8 infected and BAL cells were stained with GR1-FITC, F4/80–Alexa Fluor 647, and TRAIL PE or isotype PE mAbs, respectively. GR1<sup>int</sup>F4/80<sup>+</sup> cells were gated and analyzed for TRAIL surface expression (left). Quantitative analysis of the proportion of TRAIL<sup>+</sup> from F4/80<sup>+</sup> BALF cells gained from PR/8-infected WT (filled bars) or CCR2<sup>−/−</sup> mice (empty bars) on days 5, 8, and 11 pi (right). (B) FACS analysis of TRAIL expression on BALF NK cells (SSC low NK1.1<sup>+</sup>), neutrophils (GR1 high F4/80<sup>+</sup>), CD8<sup>+</sup> T cells (SSC low CD8<sup>+</sup>), and CD4<sup>+</sup> T cells (SSC low CD4<sup>+</sup>) of PR/8-infected WT (top) or CCR2<sup>−/−</sup> (bottom) mice gained at the indicated time points. Values are the means ± SD from two to four mice per group from at least two independent experiments. ***, P < 0.005.

Figure 7. x31-infected WT and CCR2<sup>−/−</sup> mice lack TRAIL expression on exudate macrophages and reveal equal levels of AEC apoptosis and alveolar barrier function. (A) Quantification of BAL exudate mononuclear phagocytes from Pappenheim-stained cytocentrifuge preparations in x31-infected WT (filled bars) or CCR2<sup>−/−</sup> (empty bars) mice. (B) Alveolar leakage of uninfected (day 0) or x31-infected (day 7) WT (filled bars) or CCR2<sup>−/−</sup> (empty bars) mice. (C, right) FACS analysis of apoptotic AEC type I from mock- or x31-infected WT or CCR2<sup>−/−</sup> mice on day 7 pi. (C, left) Quantification of FACS data. Bar graphs show the annexin V<sup>+</sup> proportion of CD45<sup>−</sup>T1α<sup>+</sup> cells. (D) GR1<sup>int</sup>F4/80<sup>+</sup> BAL cells from x31-infected WT or CCR2<sup>−/−</sup> mice were gated and analyzed for TRAIL surface expression. *, P < 0.05; ***, P < 0.005. All bar graphs represent the means ± SD from three independent experiments.
respectively; unpublished data), suggesting that exudate macrophages recruited to the alveolar space in WT but less in CCR2^{-/-} mice were not the primary source of TNF-α. No significant up-regulation of FasL mRNA could be detected in the analyzed cell populations (Fig. 5 B, middle). In contrast, TRAIL mRNA was selectively up-regulated fourfold in exudate macrophages as compared with their peripheral blood precursors from either mock- or PR/8-infected mice (Fig. 5 B, right).

TRAIL expression in the alveolar space is largely restricted to alveolar exudate macrophages during PR/8 infection

To evaluate TRAIL protein expression on the surface of alveolar mononuclear phagocytes, BALF cells from mock- or PR/8-infected WT or CCR2^{-/-} mice were analyzed by flow cytometry for F4/80 and TRAIL coexpression by day 8 pi. TRAIL was exclusively found on F4/80^{+} BALF cells from PR/8-infected WT but not CCR2-deficient mice, indicating that only CCR2 dependently recruited exudate macrophages but not resident alveolar macrophages expressed TRAIL (Fig. 6 A, left). The proportion of TRAIL^{+} alveolar mononuclear phagocytes (F4/80^{+}) raised to \( \sim 14\% \) in PR/8-infected WT mice on day 8 pi and was always <1.5% in CCR2-deficient mice in the time course of infection (Fig. 6 A, right). As opposed to previous reports (18), in our model, TRAIL was only expressed on a small proportion of alveolar NK cells (WT, 5.6%; CCR2^{-/-}, 5.2%) and was absent on alveolar CD4^{+} and CD8^{+} T cells as well as on alveolar neutrophils from both WT and CCR2^{-/-} mice at the indicated time points (Fig. 6 B). In addition, TRAIL was undetectable on each of these leukocyte populations including monocytes in peripheral blood (unpublished data).

Absence of TRAIL on alveolar exudate macrophages in x31-infected mice is associated with preservation of alveolar barrier function

Given that PR/8 is highly virulent in mice, we questioned whether exudate macrophages would also act pro-apoptotically toward AECs upon a less severe viral lung infection. Therefore, we infected WT and CCR2-deficient mice with a less pathogenic IV strain, x31, at a nonlethal dose. Upon x31 infection, exudate macrophages accumulated in the alveolar air spaces of WT mice (yet to a lower extent than upon PR/8 infection) and were significantly reduced in the BALF of CCR2^{-/-} mice (Fig. 7 A). However, in contrast to PR/8 infection, x31 induced only a mild alveolar leakage on day 7 pi without differences between WT and CCR2-deficient mice (Fig. 7 B). Accordingly, AEC type I apoptosis was present on day 7 pi (\( \sim 25\% \)) but no differences were detectable between the two mouse strains (Fig. 7 C). Interestingly, this was correlated to the absence of TRAIL expression on F4/80^{+} alveolar mononuclear phagocytes in x31-infected WT mice (Fig. 7 D), indicating that the induction of TRAIL on exudate macrophages is linked to more severe alveolar inflammation as observed in PR/8 infection.

Anti-TRAIL treatment attenuates alveolar epithelial apoptosis, as well as lung leakage, and enhances survival after PR/8 infection

To evaluate the contribution of exudate macrophage TRAIL to alveolar epithelial apoptosis, TUNEL assay was performed on cryosections of lavaged lungs from PR/8-infected WT mice treated with isotype IgG or anti-TRAIL mAb. Alveolar exudate macrophage numbers in anti-TRAIL- or IgG-treated mice were comparable to those of untreated mice upon PR/8 infection (IgG, 1.97 ± 0.25 \times 10^{5}; anti-TRAIL, 1.83 ± 0.77 \times 10^{5}). As shown in Fig. 8 A, alveolar cell apoptosis was pronounced in isotype IgG-treated mice but significantly decreased in anti-TRAIL-treated mice on day 7 after PR/8 infection. In addition, anti-TRAIL treatment significantly reduced annexin V binding of AEC type I in PR/8-infected lung homogenates as compared with isotype IgG-treated controls (Fig. 8 B). Analysis of alveolar barrier function in anti-TRAIL-treated PR/8-infected WT mice revealed a significant decrease in alveolar leakage in comparison to isotype IgG-treated control mice on day 7 pi (Fig. 8 C). Reduction of alveolar leakage in anti-TRAIL-treated mice was associated with significantly increased survival of IV infection as compared with isotype-treated controls (Fig. 8 D), although viral clearance from PR/8-infected lungs was substantially delayed upon anti-TRAIL treatment in comparison to untreated WT mice (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20080201/DC1). These data clearly demonstrate a crucial role of TRAIL expressed by alveolar exudate macrophages, as opposed to direct viral cytopathic effects, in the development of alveolar barrier dysfunction as a major determinant of mortality during IV pneumonia in mice.

Interestingly, TRAIL receptor (DR5) mRNA transcripts were found to be present in cultured primary murine AEC and were up-regulated upon PR/8 infection in a dose-dependent manner in vitro (Fig. 8 E). Moreover, DR5 was present on type I AEC and further up-regulated on the infected (NP^{+}) proportion on day 5 pi in vivo (Fig. 8 F, left). Increased DR5 expression persisted on NP^{+} compared with NP^{-} type I AEC until NP detection was lost by day 14 pi (Fig. 8 F, right), suggesting an enhancing effect of viral infection for macrophage TRAIL-mediated alveolar injury.

AEC apoptosis and leakage are attenuated in PR/8-infected mice recruiting TRAIL-deficient exudate macrophages to the alveolar air space

To strengthen the hypothesis that alveolar exudate macrophage accumulation mediates TRAIL-induced injury in PR/8 pneumonia, the following BM chimeric mice were PR/8-infected and were up-regulated upon PR/8 infection in a dose-dependent manner in vitro (Fig. 8 E). Moreover, DR5 was present on type I AEC and further up-regulated on the infected (NP^{+}) proportion on day 5 pi in vivo (Fig. 8 F, left). Increased DR5 expression persisted on NP^{+} compared with NP^{-} type I AEC until NP detection was lost by day 14 pi (Fig. 8 F, right), suggesting an enhancing effect of viral infection for macrophage TRAIL-mediated alveolar injury.
with TRAIL^{+/−} BM, with mice of all transplantation groups recruiting comparable alveolar exudate macrophage numbers (not depicted). Inhibition of alveolar exudate macrophage recruitment by anti-CCR2 antibodies significantly reduced alveolar leakage in mice after transplantation of WT (TRAIL^{+/+})
but not in mice after transplantation of TRAIL\textsuperscript{−/−} BM. These data indicate that TRAIL expression on grafted and then CCR2 dependently recruited cells rather than on lung resident cells is linked to lung injury. In addition, in an adoptive transfer approach using CCR2\textsuperscript{−/−} recipient mice that were either transferred TRAIL\textsuperscript{+/+}/CCR2\textsuperscript{−/−}, TRAIL\textsuperscript{−/−}/CCR2\textsuperscript{+/+}, or TRAIL\textsuperscript{+/+}/CCR2\textsuperscript{+/+} mononuclear cells, alveolar leakage was assessed upon PR/8 infection. In this model, CCR2-deficient recipient mice will largely recruit adoptively transferred CCR2\textsuperscript{+/+} monocytes into their lungs. As shown in Fig. 9 C, alveolar leakage at day 7 pi was significantly less in mice recruiting TRAIL-deficient compared with mice recruiting TRAIL\textsuperscript{+/+} exudate macrophages, with comparable alveolar exudate macrophage numbers in both treatment groups at day 7 pi (TRAIL\textsuperscript{+/+}/CCR2\textsuperscript{+/+} MNC, 5.79 ± 1.02 × 10\textsuperscript{5}; TRAIL\textsuperscript{−/−}/CCR2\textsuperscript{+/+} MNC, 4.87 ± 1.48 × 10\textsuperscript{5}). Recipient mice that were transferred CCR2\textsuperscript{−/−} MNC recruited only 1.62 ± 0.99 × 10\textsuperscript{5} exudate macrophages and correspondingly demonstrated low leakage. These data clearly demonstrate that TRAIL-induced lung injury in PR/8 pneumonia is mediated by alveolar recruitment of TRAIL\textsuperscript{+/+} but not TRAIL\textsuperscript{−/−} exudate macrophages.

**DISCUSSION**

IV pneumonia is characterized by the rapid development of acute lung injury with a poor outcome. One of the hallmarks of acute lung injury is an incremental alveolar barrier dysfunction followed by accumulation of protein-rich edema fluid in the alveolar compartment (12, 26, 27). Both viral pathogenicity factors and imbalanced host immune responses have been attributed a role in IV-induced lung failure (28–30). However, the molecular cross talk between immune and lung structural cell populations that leads to severe immunopathology and organ dysfunction is largely unknown.

Therefore, we tested the hypothesis that exudate macrophages, recruited to the lung in a CCL2/CCR2-dependent manner, might contribute to lung barrier dysfunction during lethal IV infection. In fact, alveolar barrier function, which is severely affected in IV-infected WT mice, was found to be significantly improved in CCR2-deficient mice exhibiting defective exudate macrophage recruitment. Consequently, CCR2 deficiency was associated with drastically decreased mortality during experimental IV pneumonia. Moreover, the pronounced lung leakage observed in WT mice correlated with an increased AEC apoptosis rate found to be induced by TRAIL, which is predominantly expressed in exudate lung macrophages. Finally, treatment of IV-infected WT mice with an anti-TRAIL mAb or use of mice recruiting TRAIL-deficient exudate macrophages not only attenuated alveolar leakage but significantly reduced mortality from IV pneumonia. Collectively, these results demonstrate that macrophage recruitment to IV-infected lungs in mice exerts detrimental effects on lung parenchymal cells of the highly sensitive gas exchange compartment severely affecting alveolar barrier function via TRAIL-induced epithelial apoptosis.

CCR2 is known to play a crucial role in host defense against pathogens. Interacting with its primary ligand CCL2 (MCP-1), CCR2 mediates the egress of GR1\textsuperscript{+} monocytes from the BM and their recruitment from peripheral blood to sites of infection (1, 23, 24, 31–33). In our previous studies, we demonstrated that monocyte transmigration upon epithelial PR/8 infection in vitro was strictly dependent on the CCL2-CCR2 axis (4). Thus, we assumed that the reduced lung leakage and mortality observed in CCR2-deficient mice during IV infection might be attributed to abrogated pulmonary macrophage accumulation. In fact, as demonstrated by the use of chimeric mice, loss of alveolar barrier function was solely dependent on CCR2 expression on circulating monocytes, indicating that mononuclear phagocyte traffic was critically involved and that CCR2-positive resident lung cell populations did not contribute to increased lung permeability in our model. Interestingly, a reduction of lung macrophage recruitment by 50% resulted in an approximately fivefold decrease in alveolar leakage, suggesting that alveolar damage only occurs beyond a critical threshold of macrophage accumulation and that partial inhibition of macrophage recruitment during IV pneumonia may strongly attenuate lung injury while presumably maintaining critical macrophage host defense functions.

Several authors demonstrated both release of GR1\textsuperscript{+} monocytes from the BM into the circulation and their inflammatory organ recruitment to be CCR2 dependent, with CCR2\textsuperscript{−/−} mice exhibiting diminished peripheral blood monocyte counts (32, 33). Accordingly, GR1\textsuperscript{+} peripheral blood monocytes were revealed to be ~50% less in PR/8-infected CCR2-deficient mice and in anti-CCR2 mAb-treated mice in our study (unpublished data), confirming the aforementioned data and supporting the concept of CCR2 involvement in distinct steps of monocyte homeostasis under inflammatory and noninflammatory conditions. Additionally, direct antibody-mediated cytotoxicity might account for reduced GR1\textsuperscript{+} blood monocyte numbers in anti-CCR2 mAb-treated mice (34). Therefore, a contribution of reduced circulating monocyte numbers to the attenuation of acute lung injury in IV pneumonia cannot be totally excluded.

CCR2 deficiency was found to be associated with a slight but significant delay in viral clearance, confirming observations reported previously by Dawson et al. (1) who suggest delayed adaptive immune responses as the underlying mechanism. However, we were not able to detect significant differences in the alveolar accumulation of CD4\textsuperscript{+} or CD8\textsuperscript{+} T lymphocytes despite the observed delay in DC recruitment, which is known as the major APC population. Ongoing IV replication in CCR2\textsuperscript{−/−} mice seemingly had no effect on alveolar epithelial integrity. In fact, epithelial apoptosis occurred when exudate macrophages were alveolar recruited in PR/8-infected WT mice between days 7 and 14 pi. This correlated with the bulk of mortality that was observed when virus titers were already reduced ~100-fold and IV nucleoprotein detection in AECs declined. These data support the concept that macrophage-mediated immune pathology accounts for the severity of PR/8-induced lung injury in WT mice. Correspondingly, in a milder nonlethal model of IV
infection (x31) where exudate macrophage numbers were 
~75% lower than in PR/8-infected WT mice, lung injury 
was considerably less pronounced, emphasizing the predomi-
nant role of host immune responses in IV-induced pathology.

Alveolar epithelial apoptosis has been described as a com-
mon feature of acute lung injury caused by direct or indirect 
factors such as pneumonia, aspiration, sepsis, or trauma (13–15).
Apoptosis is a regulated form of cell death that is mediated by 
membrane death receptors (extrinsic pathway) and direct mi-
tochondrial injury (intrinsic pathway). Apoptosis has been 
described to occur in the lungs of patients with acute lung 
injury by activation of the epithelial membrane Fas death re-
ceptor by soluble FasL, released from invading leukocytes 
and accumulating in a biologically active form at the onset of 
lung injury (35, 36). However, in our model of IV-induced 
acute lung injury, FasL transcripts were not differentially reg-
ulated in the macrophage subsets analyzed. In contrast, the 
proapoptotic molecule TRAIL not expressed in peripheral 
blood monocytes was found to be strongly up-regulated in 
exudate macrophages recruited into the alveolar space in IV-
infected WT mice, whereas TRAIL was undetectable in the 
alveolar mononuclear phagocytes of IV-infected CCR2−/− 
mice. TRAIL induction in alveolar exudate macrophages 
may be caused by the recruitment process into the lung itself 
or by inflammatory mediators present within the IV-infected 
alveolar compartment. Notably, TRAIL induction on re-
cruted macrophages was observed in PR/8 infection, repre-
senting a very severe and highly inflammatory form of IV 
pneumonia in mice, but not in x31 infection, suggesting that 
TRAIL-mediated alveolar injury might be restricted to se-
vere forms of IV pneumonia as observed during human 
H5N1 infections. Consistent with this, previous reports iden-
tified type I IFNs as potent inducers of TRAIL in monocytes 
and DCs (21, 37). Indeed, we found IFN-α to be alveolar 
released upon PR/8 infection in WT mice yet peaking early 
by day 2 pi (219 ± 78 pg/ml; unpublished data). TRAIL, 
acting as a membrane-bound or soluble form, has been 
known to induce apoptosis in various tumor cells but not in 
nonneoplastic cells (20, 21). Recently, TRAIL expressed 
on lymphocyte subpopulations, DCs, and monocyte-derived 
macrophages has been associated with antiviral host responses 
during IV infection (17, 18, 38), underlining the importance 
of death pathways in antiviral immunity. In contrast, Wurzer 
et al. (39) found TRAIL-induced programmed cell death 
pathways to be crucial for IV propagation in vitro. In mice, 
TRAIL exclusively acts via the membrane DR5 that is ex-
pressed on the target cell. Interestingly, DR5 was up-regulated 
in primary murine AECs in vitro and in vivo upon PR/8 in-
fec tion, suggesting that a preceding epithelial IV infection 
might facilitate TRAIL-induced apoptosis as a potential host 
mechanism to limit viral spread. Indeed, in our model, PR/8 
clearance revealed to be delayed in mice upon anti-TRAIL 
treatment. However, DR5 expression was also detectable in 
uninfected epithelial cells, indicating that exudate macro-
phage TRAIL may have the potential to attack noninfected 
epithelial cells as well when released in large amounts.

Consistent with this, the number of infected (NP+) AECs 
was only slightly reduced in WT compared with CCR2−/− 
mice on days 11 and 14 pi, indicating only partial selectivity 
of TRAIL-mediated killing between infected and noninfected 
cells. These data suggest that, upon such conditions, the de-
structive effects of TRAIL prevail over its antiviral function 
thereby leading to enhanced lung injury and mortality. 

A recent study (40) confirms the concept that monocyte-
derived cells significantly contribute to immunopathology 
and lung injury in PR/8 pneumonia, but the authors suggest 
the underlying mechanism to be an increased production of 
dindiculable nitric oxide synthase and TNF-α. Regarding TNF-
α release, we could not detect significant differences in alve-
olar-secreted TNF-α during the time course after PR/8 infection 
in WT and CCR2−/− deficient mice (unpublished data). As 
previously published by Akaike et al. (41), reactive oxygen 
and nitrogen species have been detected in the alveolar fluid 
of IV infected mice, and macrophages and neutrophils were 
identified as the primary source. Although Lin et al. (40) did 
not provide direct evidence that monocyte/macrophage-de-
derived reactive nitrogen species accounted for the observed 
lung damage in their model, it is conceivable that macro-
phage-released proinflammatory mediators additionally con-
tribute to lung injury in IV pneumonia.

Altogether, our study demonstrates for the first time a key 
role of exudate macrophage TRAIL in AEC apoptosis promot-
ing alveolar barrier dysfunction during IV lung infection. No-
tably, inhibition of TRAIL activity largely protected mice from 
lethal IV pneumonia. Targeting the TRAIL–DR5 pathway 
might therefore be a suitable tool for restoring lung epithelial 
function in patients suffering from severe IV pneumonia.

MATERIALS AND METHODS

Reagents. The following anti-mouse mAbs/secondary reagents were used for flow cytometry: CD45.2-FITC and CD45.2-PerCP-Cy5.5 (30F-11), CD45.1-PE (A20), GR-1-PE and GR-1-FITC (RB6-8C5), GR-1-PE-Cy7 (RB6-8C5), biotinylated 1-A1-E (2G9), NK.1.1-APC (PK136), CD4-FITC (RM4-4), biotinylated CD8α (53-6.7), CD11c-PE (HL3), CD11b-FITC (M1/70), SA-APC-Cy7, biotinylated F(ab)2; anti-mouse Ig, Streptavidin-PE, isotype-matched control IgG antibodies (all BD), F4/80–Alexa Fluor 647 (CF:A3-1; Invitrogen), F4/80–Alexa Fluor 488 (BMS; Invitrogen), CD11c-PE-Cy5.5 (418; Invitrogen), CD11b-PE (MCA1898; AbD Serotec), TNFα/Podoplanin/gp36 (Abcam), TRAIL-PE (N2B2; BioLegend), TRAIL R2 (DR5)–PE (118929; R&D Systems), isotype-PE (rat IgG2a; BioLegend), antinfluenza NP (Meridian Life Science, Inc.), annexin V–Al-
lex Fluor 647, anti-hamster Ig Alexa Fluor 488, and anti-mouse Ig Alexa 
Fluor 647 (all Invitrogen). 150 μg of low endotoxin/azide-free anti-TRAIL 
anti-mAb (N2B2; IgG2a; BioLegend) in 150 μl PBS−/− was applied i.p. at days 
3, 5, 7, and 9 pi, and 75 μg of rat anti-mouse CCR2-2 mAb (IgG2b; gift from 
M. Mack, University of Regensburg, Regensburg, Germany) in 100 μl PBS−/− was applied i.p. at days 0 and 4 pi (42). Representative isotype control 
IgG Abs (IgG2a [BioLegend] and IgG2b [BD]) were applied at the same 
concentrations. Propidium iodide was purchased from Sigma-Aldrich.

Mice. C57BL/6 WT mice were purchased from Charles River Laborato-
ries. CCR2-deficient mice were generated as described previously and back-
crossed to the C57BL/6 background (43). B6.SJL-Ppy−/− mice expressing the 
CD45.1 alloantigen (Ly5.1 PTP) on circulating leukocytes (C57BL/6 genetic 
background) were obtained from The Jackson Laboratory. TRAIL-
deficient mice (C57BL/6 genetic background) were a gift from Amgen.
Mice were bred under specific pathogen-free conditions. All experiments were approved by the regional council (Regierungspäanzium) of Giesen.

**Treatment protocols.** Mice were intratracheally inoculated with 500 PFU of IV PR/8 (A/PR/8/34; H1N1) or with 50,000 PFU of A/HKx31 (x31; H3N2) diluted in sterile PBS to a total volume of 70 µl mock-infected with PBS alone, and blood and BAL leukocytes were gained and processed as previously outlined in detail (44). BAL cells were counted with a hemocytometer, and differential cell counts of Pannephinem-stained cytospin preparations were performed using overall morphological criteria, including differences in cell size and shape of nuclei. Alveolar leakage in treated mice was analyzed by the lung permeability assay by i.v. injection of FITC-labeled albumin as previously described (2). For apoptosis detection, perfused lungs from infected or mock-infected mice were digested as previously outlined in detail (4), washed, and resuspended in FACS buffer (PBS, 7.4% EDTA, and 0.5% FCS). For quantitative analysis of interstitial exudate macrophages, lavaged and perfused lung tissue from WT or CCR2-deficient mice was prepared as previously described (2) and resuspended in FACS buffer.

**Lung virus titers.** Lungs were mechanically homogenized in 2 mL PBS at 1000 rpm on ice and centrifuged at 4,000 rpm for 10 min at 4°C, and supernatants were serially diluted (1:10^0 to 1:10^7 ) in PBS/BA containing 0.2% BSA, 1 mM MgCl₂, 0.9 mM CaCl₂, 100 U/ml of penicillin, and 0.1 mg/ml of streptomycin. Virus titers were determined by immunohistochemistry on confluent Madin-Darby canine kidney cells in 96-well plates in duplicates. In brief, cells were incubated with 50 µl of homogenate dilution for 1 h at room temperature and covered with 1.5% methylcellulose media containing 2 µg/ml trypsin (PAA) for 72 h. Cells were permeabilized and fixed with PBS/4% PFA/1% Triton X-100, incubated with diluted primary anti-PR/8 nucleoprotein mAb (Biozol) and secondary HRP-conjugated anti-mouse antibody for 45 min each, and stained with an AEC staining kit (Sigma-Aldrich) for 10 min. Foci were counted using a light microscope (DM 2500; Leica).

**Flow cytometry and cell sorting.** 1–5 × 10⁶ PFA-fixed cells were washed in either FACS or annexin V staining buffer (10 mM Hepes, 140 mM NaCl, and 2.5 mM CaCl₂) and permeabilized with the respective primary and secondary Abs, and flow cytometric analysis was performed using a FACSCanto flow cytometer (BD) as previously described (2). Cells stained with primary CCR2 mAb were coupled with biotinylated F(ab)², anti-Ig for 30 min before a 15-min incubation with Streptavidin-PE. For cell sorting experiments, unfixed blood or BAL cells from eight mice per experiment were incubated with the given Abs and high purity sorted with a FACSVantage SE flow cytometer as previously outlined in detail (2). Purities of sorted cell populations were always >90%.

**ELISA.** Cytokine levels from BALF were analyzed using commercially available ELISA kits (R&D Systems) according to the manufacturer's instructions. Detection limits were 2 pg/ml for CCL2, 5.1 pg/ml for TNF-α, and 12.5 pg/ml for IFN-α.

**Creation of BM chimeric mice.** BM cells were isolated under sterile conditions from the tibiae and femurs of WT C57BL/6 or from CCR2-deficient or TRAIL-deficient donor mice as described previously (25). In some experiments, WT and CCR2-deficient BM cells were mixed at a 1:1 ratio before transplantation. As controls for BM engraftment, WT C57BL/6 BM cells (expressing the CD45.2 alloantigen) were transplanted into CD45.1 alloantigen-expressing C57BL/6 mice (n = 2 during each transplantation experiment), and the proportion of CD45.2-positive peripheral blood leukocytes was analyzed by flow cytometry. BM engraftment was 90.5 ± 2.7% 2 wk after transplantation. Chimeric mice were housed under specific pathogen-free conditions for 14 d before PR/8 infection.

**Preparation of lung tissue sections and TUNEL assay.** Perfused lungs were carefully lavaged with 400-µl aliquots of PBS to wash out alveolar leukocytes and were thereafter slowly inflated with 1.5 ml of a 1:1 mixture of Tissue-Tek OCT (Sakura) and PBS alone, removed en bloc, and snap frozen in liquid nitrogen. Lung tissue cryosections were mounted on glass slides and dried overnight at room temperature. Apoptotic cells were stained using the DeadEnd Colorimetric TUNEL system (Promega) according to the manufacturer's instructions. Slides were analyzed with a light microscope (DM 2000; Leica) at the indicated magnification using digital imaging software (Leica).

**RNA isolation and real time RT-PCR.** RNA from sorted blood or BAL cells was isolated using the RNeasy Micro kit (QIAGEN), whereas RNA from cultured AECs was isolated by PeqGOLD Total RNA kit (PEQLAB) according to the manufacturer's instructions. For cDNA synthesis, reagents and incubation steps were performed as described previously (2). Reactions were performed in an ABI 7900 Sequence Detection System (Applied Biosystem) using SYBR–Green I as fluorogenic probe in 25-µl reactions containing 5 µl cDNA sample, Platinum SYBR Green qPCR SuperMix (Invitrogen), and 45 pmol of forward and reverse primers. The following primer sequences were used: TNF-α forward, 5'-CATCTTCCTCAA-AATTCGAGTGACAA-3', and reverse, 5'-TGGGAGTAGACAAGGTG TACAACCC-3'; FasL forward, 5'-CCAACAAAGCCTTAATGATCTATCATC-3', and reverse, 5'-AACCCGTTTTGGTGTAGCACAA-3'; TRAIL forward, 5'-GAAGACCTCGAAAGTGGC-3', and reverse, 5'-GACCGACTTCTACCTCATTCTCA-3'; D35 forward, 5'-AAGTGTGTCCTCCA AACGCG-3', and reverse, 5'-AATGCACAGAGTTCGCATCATC-3'; and PBGD forward, 5'-GGTACAAAGCTTCTACGACTGC-3', and reverse, 5'-ATTCGGTAAACGCGCGC-3'. Relative expression was determined by the 2^ΔΔct method.

**AEC isolation and infection.** AECs were isolated from untreated WT mice, cultured for 5 d, and infected with influenza A virus at the indicated multiplicity of infection for 8 h as previously described in detail (4).

**Adoptive transfer of PBMC.** For isolation of PBMCs, whole blood cells gained from WT, TRAIL-deficient, or CCR2-deficient mice were carefully layered over 3 ml Lympholyte (Biozol). Cells were centrifuged at 1,400 rpm at room temperature for 35 min to separate the mononuclear fraction. The interphase was collected and mononuclear cells were washed twice in RPMI 1640. 2 × 10⁶ MNC in 150 µl RPMI were injected i.v. directly before PR/8 infection into CCR2-deficient mice. Purity of MNC was always >90%, as assessed by differential counts on Pappenheim-stained cytospin preparations.

**Statistical analysis.** All data are given as the mean ± SD. For analysis of statistical differences, one-factor ANOVA was applied. Statistical significance between treatment groups were calculated with the SPSS for Windows software program (SPSS, Inc.). Significance was assumed when P values were <0.05.

**Online supplemental material.** Fig. S1 shows the time course of intrastitial lung exudate macrophage proportions in PR/8-infected WT and CCR2-deficient mice. Fig. S2 shows the time course of viral titers in lung homogenate of PR/8-infected untreated or anti-TRAIL–treated WT mice. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20080201/DC1.

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