Alveolar Epithelial Cells Direct Monocyte Transepithelial Migration upon Influenza Virus Infection: Impact of Chemokines and Adhesion Molecules

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Influenza A virus pneumonia is characterized by severe lung injury and high mortality. Early infection elicits a strong recruitment of monocytes from the peripheral blood across the endo-/epithelial barrier into the alveolar air space. However, it is currently unclear which of the infected resident lung cell populations, alveolar epithelial cells or alveolar macrophages, elicit monocyte recruitment during influenza A virus infection. In the current study, we investigated whether influenza A virus infection of primary alveolar epithelial cells and resident alveolar macrophages would elicit a basal-to-apical monocyte transepithelial migration in vitro. We found that infection of alveolar epithelial cells with the mouse-adapted influenza A virus strain PR/8 strongly induced the release of monocyte chemoattractants CCL2 and CCL5 followed by a strong monocyte transepithelial migration, and this monotypic response was strictly dependent on monocyte CCR2 but not CCR5 chemokine receptor expression. Analysis of the adhesion molecule pathways demonstrated a role of ICAM-1, VCAM-1, integrin-associated protein (CD47), and junctional adhesion molecule-c on the epithelial cell surface interacting with monocyte β1 and β2 integrins and integrin-associated protein in the monocyte transmigration process. Importantly, addition of influenza A virus-infected alveolar macrophages further enhanced monocyte transmigration across virus-infected epithelium in a TNF-α-dependent manner. Collectively, the data show an active role for virus-infected alveolar epithelium in the regulation of CCL2/CCR2-dependent monocyte transepithelial migration during influenza infection that is essentially dependent on both classical β1 and β2 integrins but also junctional adhesion molecule pathways.

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Influenza A virus is a highly contagious RNA virus causing infection of the upper and lower respiratory tract. Primary viral pneumonia is the most severe complication observed during influenza infection and reveals high mortality (1). Influenza virus replicates in bronchial and alveolar epithelial cells (AEC) and infection spreads to adjacent resident alveolar macrophages (AM) (2–5). Influenza A virus infection of the lower respiratory tract is characterized by an early influx of a small number of neutrophils followed by the recruitment of large numbers of blood-derived monocytes within the first 2–3 days of infection. By day 7, CD8 CTL from mediastinal lymph nodes accumulate within inflamed lungs. In addition, the accumulation of large numbers of monocytes within the lung parenchyma and alveolar spaces has been described as a hallmark of host defense, during early stages of viral infection, to initiate adaptive immune responses (3, 6–9). However, the cellular interactions eliciting alveolar monocyte accumulation in influenza A virus-infected lungs and underlying adhesion molecule pathways are largely unknown.

The process of inflammatory leukocyte recruitment toward the lungs in response to influenza A virus infection is initiated by the release of proinflammatory cytokines like TNF-α and IL-1 along with a variety of chemokines like CCL2 (MCP-1), CCL5 (RANTES), CCL3/4 (MIP-1αβ), CXCL10 (IFN-inducible protein 10), and CXCL8 (IL-8) from infected AM and epithelial cells (4, 8–15). Particularly the CC chemokines CCL2, CCL3, and CCL5 are major monocyte chemoattractants, with CCL2 acting via the CC chemokine receptor CCR2 and CCL3 and CCL5 interacting with chemokine receptor CCR5 (16–20). It has been shown that CCR2, rather than CCR5, contributes to monocyte recruitment into the lungs of influenza A virus-infected mice (6). However, the precise molecular cross-talk between virus-infected resident lung cells, including AEC and AM and transmigrating monocytes that are recruited into the alveolar air space upon viral infection remains unclear. Moreover, the adhesion molecule interactions underlying the multistep process of monocyte transmigration across virus-infected alveolar epithelium are poorly defined.

In the current study, we have established an in vitro model of monocyte transmigration across influenza A virus-infected murine primary AEC. We demonstrate that monocyte transepithelial migration in vitro is critically dependent on engagement of the CC chemokine receptor CCR2 but not CCR5 and is mediated via β1.
(CD49d) and β2 integrin (CD11/CD18)-dependent recruitment pathways. Finally, we demonstrate that virus-induced monocyte migration is further enhanced by a TNF-α-dependent cross-talk between virus-infected AEC and AM.

Materials and Methods

Animals

Female BALB/c mice (weight 18–21g) were purchased from Charles River Laboratories. CCR2-deficient mice were generated on a mixed C57BL/6 × 129/Ola genetic background by targeted disruption of the CCR2 gene as described previously (17). CCR2-/- mice were backcrossed for six generations to wild-type BALB/c mice. CCR5-deficient mice and wild-type mice of the corresponding genetic background (B6;129P2-Ccr5<sup>−/−</sup>J and B6129P2F2J) were purchased from The Jackson Laboratory. Mice were bred under pathogen-free conditions. All experiments were approved by our local government committee of Giessen.

Isolation and culture of murine primary AEC

Primary AEC were isolated as described previously, with some modifications (21). Briefly, BALB/c mice were killed by an overdose of euthanasia and exsanguinated by cutting the inferior vena cava. Lungs were perfused with 20 ml of sterile HBSS via the right ventricle until they were visually free of blood. A small incision was made into the exposed trachea to insert a shortened 21-gauge cannula that was firmly fixed and a total volume of 1.5 ml of sterile disperse (BD Biosciences) followed by 500 µl of sterile 1% low-melting agarose in PBS (Sigma-Aldrich) was administered into the lungs. After 2 min of incubation, the lungs were removed and placed into a culture tube containing 2 ml of dispase for 40 min. Lungs were then transferred into a culture dish containing DMEM/2.5% HEPES buffer/0.01% DNase (Serva), and the tissue was carefully dissected from the airways and large vessels. The cell suspension was successively filtered, resuspended in 10 ml of DMEM supplemented with 10% FCS and antibiotics, and incubated with biotinylated rat anti-mouse CD16/32 and rat anti-mouse CD45 mAbs (BD Pharmingen) for 30 min at 37°C. Cells were then washed and incubated with streptavidin-linked MagneSphere Paramagnetic Particles (Promega) for 30 min at room temperature with gentle rocking followed by magnetic separation of contaminating leukocytes for 15 min. The purity of freshly isolated AEC contained in the supernatant was always >90%, as assessed by modified Papanicolaou and pro-surfactant protein C immunofluorescence staining specific for type II AEC as well as immunohistochemistry for cytokeratin. Viability was always >95%, as assessed by trypan blue dye exclusion. For cytokine and adhesion molecule analysis, AEC were plated into 24-well cell culture plates at a density of 5 × 10⁴ cells/well and grown to 90% confluence for 5 days in DMEM supplemented with 10% FCS and antibiotics, thereby acquiring type I epithelial cell phenotype, as verified by loss of pro-SPC staining. For transmigration assays, 3 × 10⁵ AEC were seeded onto the lower side of Transwell filter inserts (6.4-mm diameter, 8-µm pore size; BD Biosciences) and grown for 5 days until they reached 100% confluence.

Isolation of peripheral blood monocytes (PB-Mo) and resident AM

For isolation of PB-Mo, mice were sacrificed by an overdose of isoflurane and blood was drawn via the inferior vena cava. PB-Mo were collected from the lungs of untreated BALB/c mice by injection of sterile 1% EDTA (pH 7.2) until a BAL volume of 4.5 ml was recovered. Cells were washed once, resuspended in 10 ml of DMEM supplemented with 10% FCS and anti-biotics, and incubated with biotinylated rat anti-mouse CD16/32 and rat anti-mouse CD45 mAbs (BD Pharmingen) for 30 min at 37°C. Cells were then centrifuged for 5 min at 500 × g, washed once, and resuspended in 1 ml of sterile 1% EDTA tubes (Sarsted), and diluted with 3 ml of PBS without Ca²⁺/Mg²⁺. Lungs were then removed and placed into a culture tube containing 2 ml of dispase for 40 min. Lungs were then transferred into a culture dish containing DMEM/2.5% HEPES buffer/0.01% DNase (Serva), and the tissue was carefully dissected from the airways and large vessels. The cell suspension was successively filtered, resuspended in 10 ml of DMEM supplemented with 10% FCS and antibiotics, and incubated with biotinylated rat anti-mouse CD16/32 and rat anti-mouse CD45 mAbs (BD Pharmingen) for 30 min at 37°C. Cells were then washed and incubated with streptavidin-linked MagneSphere Paramagnetic Particles (Promega) for 30 min at room temperature with gentle rocking followed by magnetic separation of contaminating leukocytes for 15 min. The purity of freshly isolated AEC contained in the supernatant was always >90%, as assessed by modified Papanicolaou and pro-surfactant protein C immunofluorescence staining specific for type II AEC as well as immunohistochemistry for cytokeratin. Viability was always >95%, as assessed by trypan blue dye exclusion. For cytokine and adhesion molecule analysis, AEC were plated into 24-well cell culture plates at a density of 5 × 10⁴ cells/well and grown to 90% confluence for 5 days in DMEM supplemented with 10% FCS and antibiotics, thereby acquiring type I epithelial cell phenotype, as verified by loss of pro-SPC staining. For transmigration assays, 3 × 10⁵ AEC were seeded onto the lower side of Transwell filter inserts (6.4-mm diameter, 8-µm pore size; BD Biosciences) and grown for 5 days until they reached 100% confluence.

Viruses strain and infection of AEC or AM

Influenza A virus strain A/PR/8/34 (H1N1; PR/8) was grown in the allantoic cavity of embryonated hen eggs. Virus titer was determined by plaque assay on confluent Madin Darby canine kidney cells. AEC or AM were washed with PBS and infected with influenza A virus at a multiplicity of infection (MOI) of 1 (unless otherwise indicated) in a total volume of 100 µl of PBS containing 0.2% BSA, 1 mM MgCl₂, 0.9 mM CaCl₂, 100 U penicillin/ml, and 0.1 mg streptomycin/ml or with diluent alone (mock infection) for 1 h at room temperature. Subsequently, the inoculum was removed and cells were incubated with either DMEM or RPMI 1640 supplemented with 2% FCS and 2 µl/ml trypsin (PAA) at 37°C for the indicated time periods.

Immunofluorescence

For immunofluorescence detection of influenza virus nucleoprotein, AEC were grown on coverslips and infected with PR/8 for 5 h, washed twice with PBS/‒, and fixed for 20 min with 4% paraformaldehyde/1% Triton X-100 (in PBS) at room temperature. Fixed cells were incubated with mouse anti-influenza nucleoprotein mAb (clone AA5H; Oxford Biotechnology) and rabbit anti-mouse widespread cytokeratin mAb (DakoCyto- mation) diluted in PBS/5% BSA for 1 h. After additional washes, cells were incubated with Texas Red-conjugated donkey anti-mouse IgG (Di-anova) plus Alexa 488-conjugated goat anti-rabbit IgG (Molecular Probes) in PBS/5% BSA for 1 h, washed again, and mounted with Mowiol (Sigma-Alrich) in glycerol/H₂O supplemented with 2.5% 1,4-diazolidicyclo-2,2,2-octane (Merck). Fluorescence was visualized with an Olympus BX60 fluorescence microscope at a magnification of ×1,000.

Cytokine quantification

Cytokine levels in the supernatants of infected AEC or AM were measured using commercially available ELISA kits (R&D Systems) according to the manufacturer’s instructions. Detection limits were 2 pg/ml for CCL2 and CCL5, 1.5 pg/ml for CCL3, and 5.1 pg/ml for TNF-α.

Flow cytometry

Flow cytometric analysis was performed using a FACS Canto equipped with a FACS Diva software package (BD Biosciences). Isolated PB-Mo or infected AEC treated with 300 µl of trypsin/EDTA solution (Clonetics) were washed and incubated for 20–60 min at 4°C with the following primary Abs: rat anti-mouse CCR5, rat anti-mouse CCR2 (20), biotinylated hamster anti-mouse ICAM-1, biotinylated rat anti-mouse VCAM-1, rat anti-mouse integrin-associated protein (IAP; all BD Pharmingen), rat anti-mouse junctional adhesion molecule (JAM-1) (Serotec), or appropriate isotype controls (BD Pharmingen). Cells were then washed twice with PBS containing 5% mouse serum and incubated with the secondary reagent streptavidin-allophycocyanin (BD Pharmingen) for 2 min at 4°C or PE-labeled goat anti-rat IgG (Serotec) for 20 min at 4°C. After two further washing steps, chemokine receptor or adhesion molecule expression was analyzed in the PE or allophycocyanin channel of the flow cytometer. Adhesion molecule expression is given as mean fluorescence intensities.

Monocyte transmigration assays

For transmepithelial migration assays, virus- or mock-infected AEC grown on Transwells were incubated in 500 µl of DMEM supplemented with 2% FCS and antibiotics added to the lower compartment of 24-well ultra low cluster plates (Costar) for 32 h. In some experiments, recombinant murine CCL2 (PeproTech) or CCL5 (R&D Systems) were added to the medium of noninfected AEC. PB-Mo (4 × 10⁵) in 100 µl of RPMI 1640/10% FCS were then added into Transwell inserts to allow their transmigration through the transmembrane inserts. After 48 h of incubation, Transwell inserts were incubated in 500 µl of sterile 1% EDTA solution (Clonetics) containing 2 mM EDTA for 90 min at 37°C. Transmigrated monocytes were collected from the lower chamber with 200–500 µl of ice-cold 5 mM EDTA in PBS, then centrifuged and resuspended in 50 µl of RPMI 1640 and total cell numbers were counted in a hemocytometer. In selected experiments, either monocytes or AEC were pretreated for 30 min at room temperature with the following azide-free function-blocking mAbs, as indicated: rat anti-mouse CD44 (clone PS/2; American Type Culture Collection), rat anti-mouse CD18 (clone 2E6; American Type Culture Collection), rat anti-mouse CD11a (clone M17/4; BD Pharmingen), rat anti-mouse CD11b (clone M1/70; BD Pharmingen), rat anti-mouse IAP (clone miap301; BD Pharmingen), rat anti-mouse ICAM-1 (clone YN1/1.7.4), rat anti-mouse VCAM-1 (clone M/K-2.7), rat anti-mouse JAM-c (clone CRAM-18 F26; Serotec), or appropriate isotype controls (BD Pharmingen). For coculture transmigration experiments, AM seeded into 24-multiswell plates were infected or mock-infected and

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coclutured with infected or mock-infected AEC grown on Transwells for 32 h in 500 µl of RPMI 1640/2% FCS/antibiotics, respectively. Conditioned medium of these coinfection experiments was then transferred into 24-well ultra low cluster plates for transmigration assays across AEC monolayers, as illustrated in Fig. 7A. In selected experiments, neutralizing anti-CCL2 or anti-TNF-α Abs or appropriate isotype controls (R&D Systems) were added to the medium of each well at 6, 12, and 20 h after infection or AEC were stimulated with rTNF-α (100 ng/ml; R&D Systems) for 24 h as indicated.

Statistical analysis
All data are given as mean ± SD. For analysis of statistical differences, one-factor ANOVA with post hoc test by Dunnett or Student’s t test were applied. Statistical significances between treatment groups were calculated with the SPSS for Windows software program. Significance was assumed when p values were <0.05.

Results

Detection of PR/8 nucleoprotein in in vitro-infected AEC
Influenza virus strain A/PR/8/34 (PR/8) is known to induce viral pneumonia in mice (6). To assess whether isolated murine primary AEC are susceptible targets for influenza A virus strain PR/8 infection in vitro, AEC monolayers were exposed to PR/8 (Fig. 1A) or diluent only (Fig. 1B), fixed within the first virus replication cycle (5 h), and coincubated with fluorescent Abs specific for PR/8 nucleoprotein and the epithelial cell marker cytokeratin. Viral nucleoprotein was detectable in the nuclei of PR/8-infected but not mock-infected AEC by immunofluorescence staining, demonstrating successful influenza A virus PR/8 infection of primary isolates of AEC in vitro.

PR/8 infection of AEC promotes monocyte transepithelial migration
To investigate whether PR/8 infection of AEC provokes a basal-to-apical monocyte transepithelial migration, isolated PB-Mo were added to Transwell filter inserts containing either PR/8 or mock-infected AEC. Transmigration rates were compared with monocyte migration across mock-infected AEC driven by recombinant CCL2 or CCL5 that was added to the lower Transwell compartment. Monocyte transmigration across PR/8-infected AEC monolayers was increased 10-fold compared with transmigration across mock-infected AEC, indicating that PR/8 infection of AEC strongly induced monocyte recruitment across the infected epithelial cell barrier (Fig. 2). Monocyte transmigration across PR/8-infected AEC even exceeded monocyte migration across mock-infected AEC driven by exogenously added recombinant CCL2 or CCL5.

Monocyte transmigration induced by epithelial PR/8 infection is dependent on the CCL2/CCR2 axis
To further characterize the chemotactic factors driving in vitro monocyte transmigration across PR/8-infected AEC, we evaluated whether PR/8 infection of AEC induced the release of the major monocyte-attracting chemokines, CCL2, CCL3, and CCL5, into the supernatant. Both CCL2 and CCL5 release was induced in a time- and MOI-dependent manner, peaking at 32 h postinfection at an MOI of 1 (Fig. 3). In contrast, CCL3 was not released by epithelial cells upon PR/8 virus infection (data not shown). To further evaluate the role of epithelial-derived CCL2 or CCL5 to drive transepithelial monocyte migration, PB-Mo collected from mice lacking the respective chemokine receptors (CCR2−/− or CCR5−/−) were compared with congenic wild-type monocytes for their transmigration capacity across PR/8-infected AEC. As demonstrated in Fig. 4A, both CCR2 and CCR5 were expressed on wild-type monocytes. Lack of the CCL2 receptor on monocytes collected from CCR2−/− mice resulted in a 90% reduced monocyte transepithelial migration. Moreover, neutralization of epithelial cell-derived CCL2 by addition of CCL2-neutralizing Abs similarly reduced the monocyte transmigration. In contrast, CCR5-deficient monocytes transmigrated PR/8-infected epithelium to the same extent as wild-type monocytes (Fig. 4B).
same extent as wild-type monocytes (Fig. 4B). These data demonstrate a crucial role for the CCL2/CCR2 axis in monocyte migration across influenza A virus-infected AEC.

Influenza A virus infection up-regulates expression of adhesion molecules on primary AEC

Given that monocyte transmigration across PR/8-infected AEC slightly exceeded the monocyte migration observed across mock-infected AEC in response to recombinant CCL2 (Fig. 2), we speculated that additional phenotypic changes in PR/8-infected AEC in parallel to the observed chemokine release contributed to PR/8-induced transepithelial monocyte migration. Therefore, using FACS analysis, we analyzed the cell surface expression of the adhesion molecules ICAM-1, VCAM-1, and IAP, known to be expressed on AEC and to mediate leukocyte-epithelial interactions (22, 23). Alveolar epithelial ICAM-1, VCAM-1, and IAP expression were found to be significantly up-regulated in PR/8-infected AEC when compared with mock-infected controls (Fig. 5A). In contrast, flow cytometric expression analysis of the adhesion molecule JAM-c, known to localize in tight junctions of tracheal and bronchial epithelium (24), showed a baseline JAM-c expression on untreated AEC and no further increase upon PR/8 infection of epithelial cells (Fig. 5B).

Role of epithelial and monocyte adhesion molecules in PR/8-induced monocyte transepithelial migration

To evaluate the contribution of epithelial- vs monocyte-expressed adhesion molecules on monocyte transmigration across PR/8-infected AEC, we blocked epithelial ICAM-1, VCAM-1, IAP, and JAM-c or monocyte CD49d, CD18, CD11a, CD11b, and IAP function by blocking Abs. Monocyte transmigration across PR/8-infected AEC was significantly reduced upon pretreatment of AEC with anti-ICAM-1 (41.9 ± 20.3%), anti-VCAM-1 (27.6 ± 11.8%), or anti-IAP (42 ± 15.2%) compared with isotype controls. Moreover, monocyte transmigration was also reduced (43.7 ± 16.9%), when AEC were preincubated with anti-JAM-c mAb, indicating that JAM-c, though not up-regulated upon PR/8 infection, appears to play an important role in influenza virus-induced monocyte transepithelial migration (Fig. 6A). As shown in Fig. 6B, transmigration was also significantly inhibited when monocytes were preincubated with anti-CD49d (23.9 ± 9.8%), anti-CD18 (48.2 ± 9.2%), anti-CD11a (31.7 ± 11.3%), anti-CD11b (43 ± 13.2%), or anti-IAP (29.5 ± 7.3%) compared with isotype controls. Anti-CD49d Ab treatment inhibited monocyte transepithelial migration much stronger than anti-CD18 Ab blockade (p < 0.01). Pretreatment of either AEC or monocytes with an anti-MHC class I mAb did not inhibit the monocyte transmigration process, thus demonstrating that Ab binding to the cell surface per se did not interfere with monocyte-epithelium interaction. These data suggest that monocyte transmigration across influenza virus-infected AEC is predominantly dependent on β2 integrin/VCAM-1 interactions, but apparently β3 integrin (CD11a/CD18, CD11b/CD18) interactions with ICAM-1 as well as IAP and JAM-c are also involved in the monocyte transmigration process.

Cross-talk between AEC and AM enhances PR/8-induced monocyte transepithelial migration in vitro

Resident AM have been shown to be susceptible targets for influenza virus in vitro and to release proinflammatory cytokines upon influenza virus infection in vivo (25, 26). To evaluate a possible
role of PR/8-infected resident AM in the monocyte transepithelial migration process, we analyzed whether virus infection of primary AM elicits the secretion of CCL2 or TNF-α, thereby aggravating the driving forces for monocyte transmigration in vitro. Interestingly, we found that CCL2 was not released by PR/8-infected AM (data not shown), whereas PR/8 infection induced the release of significant amounts of TNF-α by AM (1839 ± 741 pg/ml (infected) vs 504 ± 148 pg/ml (mock infected); p < 0.05). These data clearly suggested that virus-infected AM might be candidates to amplify the monocyte transepithelial migration process due to their virus-induced inflammatory TNF-α release. To further evaluate whether PR/8 infection of AM indeed would increase monocyte transepithelial migration via TNF-α in vitro, we coinfected AM and AEC with PR/8 in vitro, as illustrated in Fig. 7A. Importantly, monocyte transmigration across PR/8-infected AEC was unchanged in the presence of mock-infected AM (104.9 ± 43%) but significantly increased in the presence of PR/8-infected AM added to the lower transmigration chamber (169 ± 45.2%; Fig. 7B, lanes 3–5). Interestingly, a similar increase in monocyte transmigration across PR/8-infected AEC was induced when PR/8-infected AEC were stimulated with recombinant TNF-α (100 ng/ml) for 24 h before monocyte transmigration (161.7 ± 40.4%; Fig. 7B, lane 6). In this line, increased transepithelial migration of monocytes observed in virus-infected AEC/AM cocultures was completely abolished when neutralizing anti-TNF-α Abs were added to the medium (105.7 ± 17%; Fig. 7B, lane 7). To further elucidate the mechanism of TNF-α–induced enhancement of monocyte transmigration, we evaluated the potential of TNF-α to induce chemokine release and adhesion molecule expression in influenza-infected AEC. Indeed, CCL2 secretion was increased 7-fold upon TNF-α treatment (Fig. 7C, left panel), and VCAM-1 expression was significantly up-regulated compared with untreated AEC (Fig. 7C, right panel), whereas ICAM-1 and IAP expression remained unchanged (data not shown). Together, these data demonstrate that a maximal monocyte transmigration was observed when both primary AEC and AM were infected with PR/8, and this effect was found to largely depend on virus-induced TNF-α release by infected AM but not on AEC-derived TNF-α, which was found to only amount to 56 ± 10 pg/ml at 32 h postinfection.

Discussion

In the present study, we show that influenza A virus infection of primary AEC elicits a basal-to-apical monocyte transepithelial migration across infected epithelium in vitro. Although virus-infected AEC displayed a pronounced release of both chemoattractants CCL2 and CCL5, monocyte transmigration across PR/8-infected epithelium was only dependent on monocyte CCR2 but not CCR5 receptor expression. Analysis of cellular adhesion molecule expression on AEC in response to PR/8 infection revealed an up-regulation of ICAM-1, VCAM-1, and IAP but not JAM-c on AEC. AEC were found to largely depend on virus-induced TNF-α release by infected AM but not on AEC-derived TNF-α, which was found to only amount to 56 ± 10 pg/ml at 32 h postinfection.
Transmigration across PR/8-infected AEC alone. C, dent experiments. PR/8-infected AEC were stimulated with rTNF-α to enhance CCL2 release and VCAM-1 up-regulation in infected AEC. PR/8-infected AEC alone (100 ng/ml, 24 h; lane 6), or were coincubated with infected AM and neutralizing anti-TNF-α Abs (1 μg/ml at 6, 12, and 20 h after co-infection; lane 7). Monocytes were then added to the filter insert to allow transepithelial migration for 90 min. Values are presented as mean ± SD for at least five independent experiments. **, p < 0.01; *, p < 0.05 for comparison with monocyte transmigration across PR/8-infected AEC alone. C. TNF-α induces enhanced CCL2 release and VCAM-1 up-regulation in infected AEC. PR/8-infected AEC were stimulated with rTNF-α (100 ng/ml) for 24 h or left untreated. CCL2 release (left panel) and VCAM-1 expression (right panel) were analyzed by ELISA and flow cytometry. Values are presented as mean ± SD for at least four independent experiments. *, p < 0.05; ***, p < 0.005.

mononuclear phagocyte trafficking in response to influenza A virus infection (6, 27). The current data add to the aforementioned study and provide evidence for an active role of virus-infected AEC to elicit monocyte transmigration in a CCL2- but not CCL5- or CCL3-dependent fashion. Interestingly, the CCL2/CCR2 axis was found to be crucial for the monocyte transmigration, as opposed to the role of CCR5. Thus, our findings demonstrate a major role of the CCL2/CCR2 axis in monocyte-epithelial interactions leading to monocyte recruitment across the influenza virus-infected alveolar epithelial barrier. The fact that transmigration of CCR5-deficient monocytes across virus-infected epithelium was not affected when compared with wild-type monocytes strongly argues against a major contribution of CCL5 to monocyte transepithelial migration in our system, which is additionally supported by the findings that CCL2 release from influenza-infected epithelial cells exceeded the CCL5 release by a factor of 3, and by the observation that monocyte recruitment across mock-infected epithelium was much weaker in response to recombinant CCL5 as compared with CCL2.

Adhesion molecule pathways involved in CCL2/CCR2-driven monocyte recruitment to the lung in vivo and transmigration in vitro have been studied extensively in bacterial infection models. In contrast, the adhesion molecule pathways that are involved in monocyte migration across virus-infected alveolar epithelial barriers are largely unknown. The adhesion molecules ICAM-1 and VCAM-1 are known ligands for β1 (CD49d/CD29; VLA-4) and β2 integrins (CD11a/CD18; CD11b/CD18), and have been shown to be up-regulated on alveolar epithelial cells in response to TNF-α stimulation or poly(I:C) treatment or during bacterial pneumonia in vitro and in vivo (18, 28–38). In addition, increased ICAM-1 and VCAM-1 expression on A549 lung epithelial cells was reported upon respiratory syncytial virus infection (39). In the present study, ICAM-1 and VCAM-1 expression was found to be up-regulated on the surface of AEC upon influenza A virus infection. Moreover, our inhibition experiments for the first time revealed that monocytes predominantly use CD49d/VCAM-1 adhesion molecule pathways to transmigrate influenza virus-infected epithelium, thereby supporting the role of VLA-4/VCAM-1 interactions as a central molecular pathway in inflammatory monocyte trafficking both in vitro and in vivo (28, 35). Since monocyte transmigration could not be blocked completely by either of the employed Abs, involvement of additional adhesion pathways like monocyte integrin interaction with extracellular matrix proteins cannot be excluded (40).

Interestingly, IAP (CD47) was found to be up-regulated in AEC upon PR/8 infection. IAP is a multiple membrane-spanning member of the Ig superfamily expressed on virtually all cell types and has been reported to promote neutrophil transmigration across endothelial and epithelial barriers after initial β2 integrin-mediated adhesion (23, 41). In the current study, we for the first time demonstrate a specific role of IAP in virus-induced monocyte transmigration. Although numerous studies suggest that both leukocyte- and epithelial-expressed IAP interact to facilitate leukocyte transmigration, the precise mechanism is largely unknown. It was recently reported that epithelial IAP functions as a ligand for the transmembrane glycoprotein signal regulatory protein α during neutrophil transmigration (42). In addition, interactions of IAP with integrins and other membrane-associated molecules on either the epithelial cell or leukocyte surface have been described previously (43), but further studies will be necessary to clarify the function of IAP in monocyte-epithelial interactions. In addition, the present study, to the best of our knowledge, for the first time demonstrates a basal expression of JAM-c on murine primary AEC. JAMs are known to be localized to both endothelial and epithelial intercellular junctions and have been reported to regulate monocyte and neutrophil transendothelial migration. In humans, JAMs are also expressed on circulating leukocytes, whereas murine neutrophils and monocytes lack JAM surface molecules (24, 44, 45). Although in our study, JAM-c expression was not found to be up-regulated in AEC upon influenza A virus infection, anti-JAM-c Abs were highly effective in blocking monocyte migration across
influenza virus-infected epithelium by ~66%. These findings suggest that JAM-c is involved in virus-induced monocyte transepithelial migration, most probably during the intercellular passage.

AM have been reported to be infected by different influenza virus strains in vivo and in vitro, resulting in the release of proinflammatory cytokines such as TNF-α, and AM are attributed a protective host defense function in influenza virus infection in vivo (2, 4). Therefore, we questioned whether resident AM would promote monocyte transcytosis during viral infection. Importantly, in the presence of PR/8-infected AM, monocyte transmigration across infected AEC was strongly increased by ~170%. At the same time, addition of PR/8-infected AM to mock-infected epithelium only slightly induced monocyte transmigration, clearly suggesting a predominant role of the alveolar epithelial barrier in the regulation of monocyte transcytosis into the alveolar air space during influenza virus infection. Mechanistically, we found that increased monocyte transcytosis in the presence of infected AM was not due to macrophage-derived CCL2 but TNF-α secretion. Thus, TNF-α secreted from virus-infected macrophages appears to be a critical determinant in the macrophage-epithelial cross-talk in influenza virus infection by inducing increased CCL2 release and VCAM-1 expression in infected epithelial cells. This concept is supported by previous reports demonstrating TNF-α to be a potent proinflammatory effector for monocyte transcytosis during stimulating the epithelial barrier (28).

In conclusion, we demonstrate that during influenza virus infection, AEC elicit the transepithelial recruitment of monocytes in a protective manner by stimulating the TNF-α secretion. Thus, TNF-α secreted from virus-infected macrophages appears to be a critical determinant in the macrophage-epithelial cross-talk in influenza virus infection by inducing increased CCL2 release and VCAM-1 expression in infected epithelial cells. This concept is supported by previous reports demonstrating TNF-α to be a potent proinflammatory effector for monocyte transcytosis in vivo. These findings suggest that JAM-c is involved in virus-induced monocyte transcytosis during the intercellular passage.

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Disclosures

The authors have no financial conflict of interest.

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