Mucosal epithelia provide strong barriers against pathogens. For instance, the outward facing apical membrane of polarized epithelial cells lacks receptors for agents, such as hepatitis C virus, herpesvirus, reovirus, poliovirus or adenovirus. In addition, macrophages eliminate pathogens from the luminal space. Here we show that human adenovirus type 5 engages an antiviral immune response to enter polarized epithelial cells. Blood-derived macrophages co-cultured apically on polarized epithelial cells facilitate epithelial infection. Infection also occurs in the absence of macrophages, if virus-conditioned macrophage-medium containing the chemotactic cytokine CXCL8 (interleukin-8), or recombinant CXCL8 are present. In polarized cells, CXCL8 activates a Src-family tyrosine kinase via the apical CXCR1 and CXCR2 receptors. This activation process relocates the viral co-receptor αvβ3 integrin to the apical surface, and enables apical binding and infection with adenovirus depending on the primary adenovirus receptor CAR. This paradigm may explain how other mucosal pathogens enter epithelial cells.
The airway epithelium is both a barrier and early detector for viruses. It involves a cohesive network of polarized cells and restricts the flux of fluids, ions and pathogens into and out of the respiratory organs. An increasing number of pathogenic human viruses are found to be transmitted between mucosal epithelia of individuals, including human immune deficiency virus HIV, influenza virus, enteroviruses, hepatitis A, B, C and E viruses, poxviruses and also human adenoviruses\(^1\). Adenoviruses are an environmental risk factor outnumbering the ubiquitous enteroviruses in surface waters\(^1\). Of the 55 human adenovirus types known today (http://www.virmlu.uzh.ch/harrach/AdVtaxlong.htm), more than one third is associated with disease. Fatal cases of adenovirus infections are mostly due to T-lymphocyte deficiencies and occur in immunosuppressed patients\(^2\). In normal individuals, respiratory epithelial cells are initial sites for infection, although the underlying mechanisms are unknown. This leads to respiratory disease, gastroenteritis, acute haemorrhagic cystitis, meningitis, encephalitis and conjunctivitis\(^3\).

Epithelial cells adhere to each other at cell junctions, which are formed by cadherins, claudins, occludins and nectins and also immunoglobulin superfamily proteins, such as CAR (cxcackievirus-adenovirus receptor) and junction adhesion molecule (JAM\(^5\)). Yet, the junctions are dynamic and allow the passage of immune cells between epithelial cells. This supports inflammatory responses\(^2\), and somehow lays receptors open for viruses like polio, herpes, adeno, cxcackie and reovirus discussed in\(^3\). As the intact epithelium is resistant to infection, it has remained unknown how viruses cross epithelial barriers.

Some pathogens are known to affect junctional integrity, as for instance, enteropathogenic Escherichia coli, or the extracellular human gastric bacteria Helicobacter pylori, which disrupt trans-epithelial resistance and re-target B1 integrin to the apical surface. Activated B1 integrin adheres to bacteria and then triggers the injection of effectors into epithelial cells, which induces proinflammatory host genes and the release of cytokines, such as interleukin-8 (CXCL8) or tumour necrosis factor-alpha (TNF-\(\alpha\)). This, in turn, attracts and stimulates immune cells to infiltrate mucosa and eventually destroy tissue integrity reviewed in ref. 11.

Viruses do not appear to drastically affect junctional integrity. Instead, some viruses directly bind to apical receptors. For example, cxcackievirus B, a picornavirus, attaches to CD55, which triggers a signalling cascade leading to virus attachment to the tight junction protein CAR (cxcackievirus B adenovirus receptor) and infectious virus entry\(^2\). Similarly, the severe acute respiratory syndrome SARS coronavirus adheres to the human angiotensin converting enzyme 2 on the apical membrane of airway epithelial cells, and infects cells by receptor-mediated endocytosis\(^3\). Likewise, sialic acid-binding viruses, such as influenza virus, polyomavirus, rotavirus or reovirus attach to the apical side of polarized epithelial cells, although the infectious uptake mechanisms are unknown\(^4\).

Here we provide evidence for a novel mechanism of epithelial infection, namely the stimulation of virus entry by virus-induced cytokines from immune cells. Our findings may shed light on how other pathogens enter cells.

Results

Macrophages enhance apical Ad5 infection of epithelial cells.

CAR localizes to the basolateral domain and tight junctions and \(\alpha\)v\(\beta\)3/5 integrins to the basal membrane of polarized airway epithelial cells, which precludes apical Ad2/5 (adenovirus type 2 and 5) binding and infection\(^5,16\). We validated this observation with two different human lung epithelial cell lines, bronchial epithelial 16HBE14o cells and alveolar type II epithelial A549 cells. When grown to confluent monolayers on transwell filters and inoculated with a non-replicating adenovirus reporter, which expresses murine interleukin (IL)-2 (Ad5-muIL2, muIL2 expression is hereafter referred to as infection)\(^17\), both cell types were poorly infected from the apical side, but were strongly infected from the basolateral side yielding infection levels of 20–40% of nonpolarized cell infections (Fig. 1a). Both cell lines expressed CAR, \(\alpha\)v\(\beta\)3- and \(\alpha\)v\(\beta\)5-integrins (Fig. 1b). Opening up the cell–cell contacts with EDTA significantly increased apical infection (Fig. 1a), consistent with results from earlier treatments of polarized cells with the divalent cation chelator ethylene-glycol-tetraacetic-acid (EGTA)\(^18\). These results were confirmed by green fluorescent protein (GFP) expression analyses from a replication-defective Ad5-eGFP in 16HBE14o cells (Supplementary Fig. S1).

Here we next assessed whether macrophages have an impact on adenovirus infection of epithelial cells. Isolated human peripheral blood mononuclear cells (PBMC)-derived macrophages could not be infected with Ad5-muIL2 up to 45 h postinfection (pi), unlike 16HBE14o cells (Supplementary Fig. S2a). As expected, PBMC-derived macrophages did not express the Ad2/5 attachment receptor CAR, and had moderate levels of \(\alpha\)v\(\beta\)3/5 integrins on their surface (Supplementary Fig. S2b). To mimic the natural situation of macrophage-controlled mucosal epithelia, we seeded macrophages onto the apical side of polarized 16HBE14o monolayers and allowed attachment for 24 h. Transmission electron microscopy of macrophage- and 16HBE14o co-cultures showed intact epithelial monolayers with macrophages attached to the apical membranes (Fig. 1c). On apical inoculation of co-cultures with Ad5 wildtype for 1 h, virions could be readily detected in vesicular structures of macrophages and to a much lower degree in epithelial cells (Fig. 1c,d). Epithelial cells cultured without macrophages contained no detectable virus particles (not shown). When co-cultures were inoculated with Ad5-muIL2 for 20 or 30 h, we detected no significant differences between apical or basolateral inoculations, whereas polarized epithelial cells without macrophages were not infected by apical inoculation (Fig. 1e). These data indicate a crucial role for macrophages in Ad5 infection of epithelial layers consisting of macrophages and epithelial cells.

Apical Ad5 infection mediated by conditioned medium.

Macrophage-mediated apical infection depends either on direct interactions of macrophages with epithelial cells, or on messengers secreted from macrophages, such as cytokines. We tested whether the supernatant from macrophages inoculated with adenovirus, the so-called conditioned medium (CM), was sufficient to increase the Ad5-muIL2 infection of polarized epithelial cells. PBMC-derived human macrophages were inoculated with Ad2 for 4 h; the medium was collected and cleared by centrifugation and added to polarized 16HBE14o cells for 4 h, followed by apical inoculation with Ad5-muIL2 for 20 or 30 h (Fig. 2a). Stimulation of polarized cells with CM led to strong increase of apical infection compared with non-stimulated cells (Fig. 2b). Although we observed variations of CM potencies between macrophages from different blood donors, the typical increase of viral infection was 8- to 12-fold. In all instances, the maximal stimulation of apical infection was obtained at 4–8 h post stimulation (Fig. 2c). Interestingly, the infection stimulation was much stronger, if CM was applied apically than basolaterally, and was only marginally increased by simultaneous addition to both apical and the basal domains compared with apical stimulation (Fig. 2d). Importantly, the CM did not lose its potency by previous centrifugation under conditions, which completely pelleted viral particles (Supplementary Fig. S3a). Heat inactivation of CM at 75 °C for 15 min reduced the stimulatory activity to almost background levels, suggesting that the heat-sensitive factor(s) were responsible for the infection-stimulating effects (Supplementary Fig. S3b). Taken together, the data suggest that adenovirus-inoculated macrophages condition the medium with heat sensitive agents, which act on the epithelial cells from the apical side and enhance apical adenovirus infection.
CM enhances apical binding of Ad2/5. We measured the trans-epithelial electrical resistance (TEER) or diffusion rate of 10 kDa dextran-fluorescein isothiocyanate (FITC) across the epithelial layer. Treatment of polarized 16HBE14o cells with CM or control macrophage supernatant did not indicate any impairment of epithelial integrity, whereas EDTA treatment drastically reduced TEER (Fig. 2e), and increased the apparent permeability of 10 kDa FITC-dextran (50 µg ml⁻¹ apically added for 30 min) to 9.7×10⁻³ cm⁻² s⁻¹ compared with 2.5×10⁻⁴ cm⁻² s⁻¹ in control or 0.5×10⁻⁶ cm⁻² s⁻¹ in CM-treated cells.

In a binding assay, we next analysed the localization of atto565-labelled Ad2 added to polarized 16HBE14o cells by incubation at 4°C. Side projections of confocal fluorescence microscopy z-stacks showed an increased apical localization of Ad2-atto565 on CM-treated cells compared with cells treated with control macrophage supernatant (Fig. 2f,g). We did not detect viral particles in basolateral areas, or within cells, suggesting that the epithelial layers were intact. We conclude that enhanced apical infection on CM treatment is not due to disrupted junctions, but rather results from changes in the apical membrane domain composition allowing for virus attachment.

CXCL8 mediates apical adenovirus infection. Adenoviruses are known to trigger the secretion of cytokines from macrophages, which coordinate a concerted antiviral immune response⁴⁹. To identify cytokines in the infection stimulating CM, we performed a BioPlex assay including 12 different cytokines with known effects on epithelial layers. At 4 h after inoculation of macrophages with adenovirus, we detected IL-6, CXCL8, IL-10 and TNF-α in the supernatant, and at 8 h, interferon (IFN-γ) was also detected, but IL-10 was no longer detected (Supplementary Table S1). We did not detect IL-1β, IL-4, IL-12/p70, IL-13, IL-15, IL-17 or IFN-α2, suggesting that these cytokines have no decisive role in stimulation of epithelial infection. The treatment of polarized 16HBE14o cells with recombinant human CXCL8 (CXCL8), however, boosted apical infection in a dose-dependent manner, although not to the full extent as CM. Recombinant human IFN-γ (IFN-γ) had no infection-supporting impact (Fig. 3a), although it increased the expression of HLA-1 (MHC-I) epitopes on A549 cells (data not shown). The depletion of CXCL8 from CM with a neutralizing antibody, however, significantly reduced the stimulation of apical infection, whereas depletion of IL-10 did not (Fig. 3b). These data suggest an important role of CXCL8 in stimulating apical adenovirus infection, although it did not account for the full stimulation by CM. This conclusion was corroborated by the finding that blocking of the CXCL8 receptors, CXCR1 and CXCR2, with neutralizing antibodies before stimulation with CM reduced CM-mediated infection to a significant extent, yet not completely (Fig. 3c). Neutralization of both receptors together did not have additive effects, suggesting a co-dependency of receptor function by heterodimerization, as earlier reviewed in ref. 20.
CXCR1 and CXCR2 are known to be internalized on ligand activation\(^{23}\), which initiates CXCL8 signalling\(^{23}\). We found that CM-stimulated polarized epithelial cells efficiently internalized both receptors within 10 min, which is consistent with a high degree of activation (Fig. 3d). Interestingly, both CXCR1 and CXCR2 were found on the apical side of polarized 16HBE14o cells by confocal imaging (Fig. 3e), supporting the finding that apical application of CM did stimulate infection of 16HBE14o cells (Fig. 2d).

**CM and CXCL8 induce apical localization of αβ3 integrin.** Our earlier results indicated that CM did not affect the integrity of the epithelial layer excluding the possibility that receptors become accessible to apical virus particles by loosened cell–cell contact areas. We next tested whether integrin receptors were expressed on the apical surface of CM-stimulated polarized epithelial cells. Flow cytometry measurements indicated that both CAR and αβ5-integrin total surface expression levels did not change on CM stimulation while the expression of αβ3 integrin was slightly decreased (Fig. 4a).

We examined the localization of αβ3 and αβ5 integrins in polarized 16HBE14o cells by confocal fluorescence microscopy. Intriguingly, side projections of z-stacks showed that αβ3 integrin was present on the apical membranes on stimulation by CM or CXCL8 (Fig. 4b), whereas αβ5 integrin remained localized to the basolateral domains (Supplementary Fig. S4a,b). The treatment of CM with neutralizing antibodies against CXCL8 before stimulation abrogated the apical localization of αβ3 integrin (Fig. 4b,c). We confirmed these results by selective biotinylation of the apical cell surface and subsequent neutravidin pull-downs of biotinylated proteins (Fig. 4d). Further analysis by SDS–PAGE and western blotting identified β3 integrin as one of the biotinylated, thus apical proteins. The membrane cofactor CD46, which was reported to localize to the apical membrane compartment\(^{23}\), was also significantly biotinylated from the apical side, similar to β3 integrin (Fig. 4d). We next tested the functional significance of the apical localization of αβ3 integrin on CM stimulation. Apical addition of anti-αβ3 integrin function blocking antibodies
to CM-stimulated 16HBE14o cells strongly reduced Ad5-muIL2 infection, whereas anti-αβ3 integrin antibodies had no significant effects (Fig. 4e).

We further confirmed the role of the receptors αβ3 integrin and CAR in apical infection on CM stimulation with competition experiments. Soluble forms of CAR and integrin receptors added after the 4 h stimulation period were able to interfere with infection in a dose-dependent manner (Fig. 4f). Control soluble CD46, however, had no effect. Likewise, soluble Ad2 fibre knob inhibited infection whereas Ad35 fibre knob, which does not bind CAR, showed no effect (Fig. 4g). In agreement with these functional interference data, we found CAR epitopes on the apical side of CM stimulated 16HBE14o cells, distinct from the tight junction marker ZO-1 (Supplementary Fig. S4c).

These results were in line with the co-localization of αβ3 integrin and CAR with Ad2-atto565 particles added apically to stimulated cells (not shown). Together, the data suggest that CM or CXCL8 enhance the localization of αβ3 integrin and CAR to the apical domain without grossly altering the overall cell surface expression of αβ3 integrin. This enables adenovirus attachment to the apical cell surface and subsequent infection.

**β3 integrin localization and apical infection are Src-dependent.** To address the nature of apical αβ3 integrin-mediated adenovirus infection, we tested the hypothesis that a mechanism akin to emerging focal complexes could be involved, which is also implicated in cell migration and wound healing. Focal contacts around integrins mediate cell adhesion to the extracellular matrix. They are dynamically regulated in migratory cells by tyrosine kinase signalling. It is of note that the chemokine CXCL8 not only acts as an important chemo-attractant to neutrophils and initiates directed cell migration, but also regulates cell migration and wound healing. Focal contacts around integrins mediate cell adhesion to the extracellular matrix. They are dynamically regulated in migratory cells by tyrosine kinase signalling. It is of note that the chemokine CXCL8 not only acts as an important chemo-attractant to neutrophils and initiates directed cell migration, but also regulates cell migration and wound healing. Focal contacts around integrins mediate cell adhesion to the extracellular matrix. They are dynamically regulated in migratory cells by tyrosine kinase signalling.

We tested whether signalling via Src and paxillin is involved in the initiation of focal complexes by signalling via Src/FAK and paxillin.

*Figure 3 | CXCL8 is an infection stimulating component of CM. (a) Recombinant human CXCL8 boosts apical infection with adenovirus. Polarized 16HBE14o cells were treated with CM, CXCL8 or IFN-γ at indicated concentrations for 4 h, or left untreated prior to apical infection with Ad5-muIL2 for 20 h. Infection was normalized against non-treated controls. Data represent means of triplicates and s.e.m.; P-value was determined by Student’s t-test. (b) Neutralizing antibody against CXCL8 significantly diminishes apical infection on CM stimulation. CM was incubated with neutralizing antibodies against CXCL8 or IL-10 before stimulation of polarized 16HBE14o cells or left untreated. Apical infection with Ad5-muIL2 was for 20 h, and normalization against non-treated controls. Experiments were carried out in triplicates. Error bars represent s.e.m. values, and P-values are from Student’s t-tests. (c) Neutralizing antibodies against CXCL8 receptors CXCR1 and CXCR2 significantly reduce apical infection on CM stimulation. Polarized 16HBE14o cells were incubated with neutralizing antibodies against CXCR1, CXCR2 alone and both together before CM stimulation. Cells were infected apically with Ad5-muIL2 and infection was normalized against untreated control. Experiments were carried out in triplicates. Error bars represent s.e.m. values, and P-values are from Student’s t-tests. (d) CXCR1 and CXCR2 are rapidly activated on CM stimulation, as indicated by reduction from the cell surface. Surface expressions of CXCR1 and CXCR2 were determined by flow cytometry in control 16HBE14o cells or 16HBE14o cells 10 min after stimulation with CM. (e) CXCR1 and CXCR2 are localized on the apical surface of polarized 16HBE14o cells. Polarized 16HBE14o cells were stained for CXCR1 or CXCR2, and ZO-1. Nuclei were stained with Hoechst. Z-projections of confocal stacks are shown. Scale bar, 10 μm.*
Figure 4 | CXCL8 is responsible for CM-induced apical translocation of αvβ3 integrin. (a) Total surface expression of adenovirus receptors CAR, αvβ3 and αvβ5 integrins determined by flow cytometry in untreated cells (black line) or after CM stimulation of confluent A549 cells for 4 h (black line, gray filled). IgG isotype control is shown in gray line. (b, c) CM and CXCL8 relocate αvβ3 integrin to the apical side. Polarized A549 cells were stimulated with CM, CXCL8 or CM incubated with neutralizing antibody against CXCL8 before stimulation. Cells were stained for αvβ3 integrin and confocal stacks were recorded. Z-projections of the stacks are shown in b. Scale bar, 15 µm. (c) Shows quantification of the mean relative fluorescence for each section of the stacks shown in b, covering an area of 246.5 µm², plotted from the apical (ap) to the basal (bl) sections. (d) Polarized 16HBE14o cells were stimulated with CM, CXCL8 or CM incubated with neutralizing antibody against CXCL8 before stimulation. Apical cell surface was biotinylated, and biotinylated proteins were pulled down with neutravidin from cell lysates. Aliquots of biotinylated samples and total cell lysate were probed for β3 integrin and CD46 on a western blot. (e) Polarized 16HBE14o cells were stimulated with CM for 4 h or left untreated. Neutralizing antibodies against αvβ3 or αvβ5 integrins were added for the last hour. Cells were apically infected with Ad5-muIL2 for 20 h. Graph shows normalized infection compared with untreated control. Data represent means of triplicates and s.e.m. (f) Competition assay with soluble forms of Fc-tagged CAR (CARex-Fc) and αvβ3 integrin (αvβ3ex-Fc) with soluble CD46 (CD46ex-Fc) as control. Polarized 16HBE14o cells were stimulated with CM for 4 h or left untreated. Soluble virus receptors were added from the apical side and incubated at 4 °C for 1 h. Cells were apically infected with Ad5-muIL2 for 20 h. Graph shows normalized infection compared with untreated control. Data represent means of triplicates and s.e.m. with P-values from Students t-tests relative to data indicated by *.

Discussion

Cells of the innate immune system, including macrophages and dendritic cells control the inflammatory response by interacting with epithelial cells. Alveolar macrophages are sensors for infection and detect adenoviruses in the airway lumen28. On contact with viruses, they undergo rapid activation and secrete pro-inflammatory cytokines, such as IL-6, CXCL8 and TNF-α26,29,30. This boosts the antiviral response, attracts leukocytes to the site of infection and critically contributes to mounting adaptive immune responses against the virus. However, activated macrophages also trigger acute lung injury and thereby exacerbate viral infection2.

Our study here identifies a mechanism, by which activated macrophages increase viral infection of polarized epithelial cells. We show that macrophages co-cultured with polarized epithelial cells on the luminal side take up virus particles and secrete cytokines, stimulated with either control medium (supernatant of non-inoculated macrophages), CM, CXCL8 or CXCL8 depleted CM for 10 min or 4 h. Strong induction of Src tyrosine 416 (Y416) phosphorylation could be detected in both CM and CXCL8-treated cells at 10 min or 4 h post treatment (Fig. 5a). Likewise, phosphorylation of paxillin on the critical tyrosine residue Y118 was induced by CM and CXCL8 at 4 h, and confirmed by immunofluorescence microscopy, which showed apical localization of pY118 paxillin (Fig. 5b; Supplementary Fig. S5a).

To test whether Src family tyrosine kinases were involved in CM-mediated apical infection, we blocked Src activity with the small compounds PP1 and PP2 (ref. 27). The treatment of polarized 16HBE14o cells with PP1 and PP2 before CM stimulation had no effects on TEER (Supplementary Fig. 5b). However, both compounds blocked CM-mediated infection at 10 µM concentrations (Fig. 5c). Western blot analysis showed that PP1 and PP2 not only inhibited Src and pY118 paxillin phosphorylation, but also blocked the apical localization of αvβ3 integrin in CM-stimulated 16HBE14o cells (Fig. 5d,e). The data suggest that Src controls β3 integrin redistribution and apical adenovirus infection (Fig. 6).
which enhance infection of the epithelial cells with adenovirus. This cytokine response does not impair the barrier function of the epithelial cells and does not require the presence of macrophages, suggesting that the infection route depends on a viral receptor in the apical epithelial membrane rather than direct transmission of viral particles from macrophages to epithelial cells, for example, a ‘Trojan-horse’ mechanism as proposed for measles virus.33

Cytokine mRNAs and proteins, including IL-6, CXCL8 are induced in response to adenovirus infection in several cell lines and also in vivo. For example, CXCL8 was elevated in bronchial alveolar lavage fluid of macaque monkeys on inoculation of an adenovirus vector encoding the cystic fibrosis gene, and thereby induced in response to adenovirus infection in several cell lines (CJ Burckhardt, M Suomalainen, P Schoenenberger, K.B., S.H. and U.F. G., unpublished results). CXCL8-driven chemotaxis can be elicited in many cell types best known for its angiogenic and neutrophil chemotactic properties. CXCL8-driven chemotaxis can be elicited in many cell types best known for its angiogenic and neutrophil chemotactic properties.33, 36, 37. CXCL8-driven chemotaxis can be elicited in many cell types expressing CXCR1/2. It has an important role in fibroblast-mediated wound healing,36, the formation of tumour metastases36, and neutrophil chemotaxis.37 Chemotaxis forms and dismantles focal complexes and adhesions, and dynamically modulates anchorage of cells to the extracellular matrix. Anchorages involves αβ3 integrin, initially in the leading edge of migrating cells. Intriguingly, CXCL8 enhancement of apical adenovirus infection required the induced localization of αβ3 integrin in the apical plasma membrane of the polarized epithelial cells, as indicated by neutralizing antibodies or soluble αβ3 integrin competition, which also blocks infection of nonpolarized cells (CJ Burckhardt, M Suomalainen, P Schoenenberger, K.B., S.H. and U.F.G., unpublished results). αβ3 integrin has an important role in viral infections, where it serves as an affinity-regulated co-receptor by binding to a 50-amino-acid RGD stretch of the adenoviral capsid protein penton base,39, 40 and also critically contributes to HIV infection of adherent macrophages.32

Chemotaxis critically involves the non-receptor tyrosine kinase family Src and paxillin, a pivotal signalling integrator and scaffold protein.41, 42, 43 Src directly binds the cytoplasmic tail of β3 integrin and is activated by αβ3 integrin, which might induce

**Figure 5** CM-stimulated apical αβ3 integrin localization and Ad5 infection are Src-dependent. (a) Src and paxillin are phosphorylated on CM stimulation. Confluent 16HBE14o cells were serum-starved for 24 h and subsequently stimulated for 10 min or 4 h with media containing serum (control), CM, CXCL8 or CM incubated with neutralizing antibody against CXCL8 before stimulation. Samples were analysed by western blot and probed for paxillin (phospho-Y118), Src (phospho-Y416) or tubulin. (b) Phospho-paxillin localizes to the apical side on CM stimulation. Polarized 16HBE14o cells were stimulated with control medium and CM and stained for paxillin (phospho-Y118), ZO-1 and nuclei (Hoechst). Confocal stacks were recorded and z-projections composed. Scale bar, 10 μm. (c) Polarization of Src-inhibitors PP1 and PP2 inhibit apical Ad5 infection on CM stimulation. Polarized 16HBE14o cells were treated with DMSO, PP1 and PP2 (10 μM) for 1 h or left untreated (DMSO). Subsequently, cells were stimulated with CM for 4 h, and infected with Ad5-mul2 for 20 h. Infection was normalized against controls. Data represent mean of triplicates and s.e.m. (d) PP1 and PP2 prevent phosphorylation of Src and reduce phosphorylation of paxillin. Confluent 16HBE14o cells were serum starved for 24 h. Cells were treated with PP1 and PP2 (10 μM) for 1 h, and stimulated with CM. Aliquots of total cell lysates were analysed by western blot and probed for paxillin (phospho-Y118), Src (phospho-Y416) or tubulin. (e) Polarized 16HBE14o cells were treated with DMSO, PP1 or PP2 (10 μM) for 1 h, and subsequently stimulated with CM for 4 h. Cells were biotinylated on the apical side, lysed and biotinylated proteins were pulled down with neutravidin. Aliquots of biotinylated samples and total cell lysate were probed for β3 integrin and CD46 on a western blot.
primary defence of epithelial barriers against infection, traumatic insults and malignancies. These cells might have pivotal roles in the defence against adenoviral infection, and if impaired contribute to the severe pathological conditions in immunocompromised individuals.

Collectively, our results uncover a novel apical infection route for adenovirus. We showed that innate immune cells release CXCL8, which triggers a signalling cascade in polarized epithelial cells resembling the induction of cell migration. During this process, Src and paxillin are tyrosine phosphorylated on critical residues. The latter is recruited to the apical membrane together with αβ3 integrin, depending on Src. In the course of this activation, the immune modulator CAR is localized to the apical membrane and becomes available for binding of virus, which together with αβ3 integrins triggers viral uptake and infection. This infection mechanism allows viral pathogens to take advantage of immunological anti-viral reactions and gain entry into a well-protected epithelium. We anticipate that other viruses whose receptors are not available for virus binding in an intact uninjured epithelium can use similar strategies to achieve entry into cells.

Methods

Cell lines and viruses. Human transformed embryonic retinoblast 911 cells were grown in DMEM medium (D6429, Sigma) supplemented with 10% FCS (Invitrogen), 1% t-glutamine and 1% non-essential amino acids (NEA, Sigma). Carcinoma alveolar type II A549 (ATCC) and transformed bronchial epithelial 16HBE14o cells obtained from Dr D. Gruener, California Pacific Medical Center Research Institute, San Francisco, USA49,50, were grown in RPMI 1640 medium (R8758, Sigma) supplemented with 10% FCS, 1% t-glutamine and 1% NEA. All cells were kept at low passage number at 37°C in a humidified chamber at 5% CO2. For microscopy of sub-confluent cells, cells were grown to 70% confluence on 18 mm glass cover slips (Menzel Gläser). Flow cytometry was performed with cells grown to 100% confluency in 24-well plate format. Ad5_CMV_eGFP and Ad2 were propagated in 911 cells and isolated and Ad2 was labeled with anti565 (Atto-TEC GmbH, Ad2-atto565) as described previously46. Ad5_CMV_mull2 was grown as described47. Virus preparations used for stimulation of macrophages were tested for possible endotoxin contaminations with Endozone Endpoint Assay Kit (Charles River Laboratories). Endotoxin levels were below 0.003 U ml−1. Soluble CAR and CD46 were produced as described in refs 56,57. Soluble αβ3 integrin was produced in COS cells transfected with cDNAs for αν and β3 integrins tagged with human Fc (1,222 amino acids for the mature αν–Fc fusion protein, and 948 amino acids for the β3–Fc), provided by Dr Curcio Ruegg (University of Fribourg, Switzerland). Fusion proteins were produced and purified as described46. Ad5 or Ad53 fibre knob proteins were produced using the Bac-to-Bac baculovirus expression system (pFastBac, Stratagene) with amino-terminal 6His-tag for single-step Ni-NTA-agarose affinity chromatography, followed by the recognition site for TEV protease. Fibre knobs comprised the residues 384 to 581 for Ad5 (GenBank: J0210559) and 126 to 323 for Ad35 (GenBank: GI5610945).

Macrophage cell culture. Macrophages were differentiated from human peripheral blood mononuclear cells as described previously46. In brief, peripheral blood mononuclear cells were isolated from buffy coats (blood donation service, Zurich, Switzerland) by density gradient centrifugation on Histopaque (Sigma). PBMCs were washed intensively in PBS and resuspended in RPMI 1640 supplemented with 1% t-glutamine, 1% penicillin/streptomycin and 10% heat-inactivated human serum (blood donation service). Cells were seeded at a density of 107 cells ml−1 and well in a 6-well plate (Nunc) and allowed to adhere for 4 h. Non-adherent cells were washed away and adherent cells cultured as described above. Macrophages were obtained without any additional supplements. Cells were kept at 37°C in 5% CO2 humidified atmosphere. Purity of differentiated cells (approximately 4×105 cells per 6-well dish) was assessed by flow cytometry with CD14 (BD Biosciences) as monocytes/macrophage marker and yielded in >95% positive cells.

Epithelial monolayers and macrophage co-culture. Establishment of epithelial monolayers and co-cultures was modified after Rothen-Rutishauser46. Briefly, epithelial cells were seeded onto cell culture inserts (3 µm pores, 24-well (353492) or 12-well (353392) format, BD Bioscience) and allowed to settle for 2 days. Seeding to filters occurred at 150% plastic dish confluency to compensate for reduction in adhesion area during the differentiation process of the epithelial cells at air–liquid interface. Medium was removed, cells were washed with PBS and subsequently kept at an air–liquid interface supplemented with medium only from the basal side (100µl in 24-wells, 250µl in 12-wells). A549 cells required 9–10 days to form a monolayer of appropriate integrity, whereas 16HBE14o could be used after 6–8 days. To obtain co-cultures, macrophages were seeded on top of epithelial cells from the apical side at a ratio of approximately 2:3, settled for 2 h, unattached...
cells rinsed off, and co-cultures (5–10 epithelial cells per macrophage) maintained at air–liquid interface for 24 h before use.

**TEER and diffusion assay.** Integrity of epithelial monolayers was assayed by measuring TEER and m apparent permeability (Perm_app). TEER was measured with an E沃M Volt-Ohmmeter (World Precision Instruments). To determine the diffusion rate, 50 µg/mL 10 kD FITC-dextran (Sigma–Aldrich) was added to the apical side and cells were incubated for 30 min. Aliquots were taken from the apical and basal side and fluorescence was measured using a Safire monochromator-based microplate detection system (Tecan). The apparent permeability (Perm_app) was calculated according to the following equation:

$$\text{Perm}_{\text{app}} = \left(\frac{dQ}{dT}\right) \times \frac{1}{A} \times \frac{1}{L} \times \frac{1}{C_p} \left(\text{cm}^2 \cdot \text{s}^{-1}\right)$$

where dQ/dT (µg per s) is the rate of transport, C_p is the initial concentration in the apical compartment (µg mL⁻¹) and A is the surface area of the filter (cm²).

**Conditioned medium.** Differentiated macrophages were incubated with 0.1 µg/µL of medium in the bottom chamber. In all other experiments, medium in both chambers was removed after 1 h and incubation proceeded for another 12 h on air–liquid interface with 100 µL of medium in the bottom chamber. In all other experiments, medium in both chambers was removed after 1 h and incubation proceeded for another 19 h on air–liquid interface with 100 µL of medium in the bottom chamber. Concentration of mll2 in the media was assessed by mll2 ELISA according to the manufacturer’s protocol (BD Biosciences). GFP-expression was visualized by confocal microscopy (CLSM (Leica SP5, Leica Microsystems)).

**Immunofluorescence and flow cytometry.** Monolayers were cultured on 12-well cell culture inserts as described above and treated with control (PBS), CM, or CCLX8 or CM preincubated with anti-CCLX8 neutralizing antibodies for 4 h. Cells were fixed with 3% paraformaldehyde (PFA), quenched with 25 mM NH₄Cl and permeabilized with 0.5% Triton X-100. Filters were washed and incubated with the first antibody at 4°C overnight. The following antibodies were used: mouse monoclonal anti-CD44 (1:100, clone 16H1, R&D Systems), mouse monoclonal anti-integrin αvβ3 (1:100, clone 16H8, Chemicon), mouse monoclonal anti-integrin αvβ6 (1:100, clone LM609, Chemicon), mouse monoclonal anti-beta-catenin (1:500, clone 14, BD Transduction Laboratories), mouse monoclonal anti-occludin (1:200, clone 19, BD Transduction), mouse monoclonal anti-ZO-1 (1:1,000, clone P1F6, Zymed), mouse monoclonal anti-fibronectin (1:1,000, clone 1BII, TaKaRa) and rabbit anti-tubulin (1:200, T13). Antibodies were diluted in TBST/5% BSA (phospho-antibodies) or TBST/2.5% dried milk (all other antibodies). HRP-conjugated secondary antibodies were detected with ECL-Plus reagents (Amersham Biosciences). Filters were stripped with 100 mM β-mercaptoethanol, 2% SDS, and 62.5 mM Tris–HCl, pH 6.7, for 30 min at 50°C, washed extensively with TBST, blocked with 5% dried milk, and reprobed with control or total antibody.

**Microscopy.** Confocal laser scanning microscopy was conducted with an inverted Leica SP5 microscope (Leica Microsystems) equipped with a 63x objective (oil immersion; NA, 1.4), a diode laser (405-nm excitation), an argon laser (485/476/488/496/514-nm excitation), and a helium laser (561/594/633-nm excitation). Image processing was performed using Image open software (http://rsweb.nih.gov/nihe/image/). Samples for transmission electron microscopy were fixed in 1.5% glutaraldehyde-2% formaldehyde in 0.1 M cacodylate buffer (pH 7.4) for 60 min, followed by postfixation in 1% OsO4 and 1.5% potassium-ferricyanide at RT for 1 h, and staining with 3% uranylacetate over-night. Samples were dehydrated with acetone, embedded in Epon, ultrathin-sectioned and analysed in a Zeiss EM10.

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Author contributions

V.L. and U.F.G. initiated the study, and U.F.G. coordinated the study. V.L. and U.F.G. performed the experiments. V.L. and U.F.G. analyzed the data and wrote the manuscript. All authors discussed the results and commented on the manuscript.

Additional information

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