Supporting Information

Macrophage-epithelial paracrine crosstalk inhibits lung edema clearance during influenza infection

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Supplemental Methods

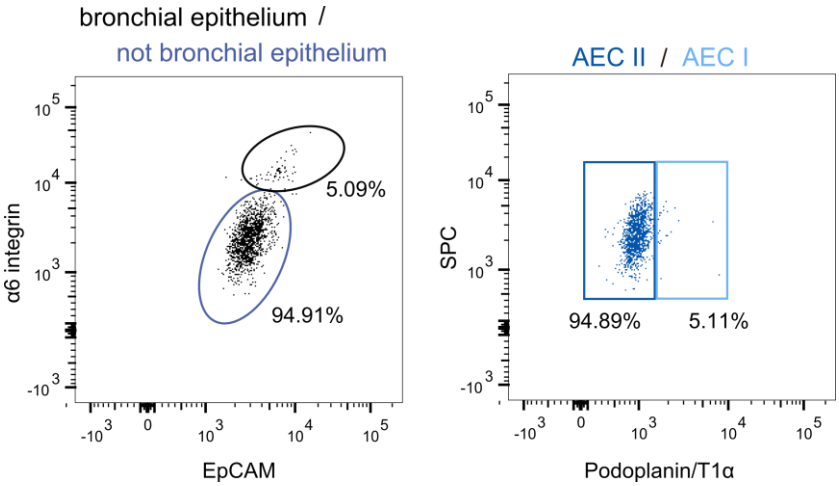
Reagents. For flow cytometric staining the following antibodies were used: Integrin α6 (Biolegend, clone GoH3), pro-surfactant protein C (Millipore) and Podoplanin (Biolegend, clone 8.1.1).

Wet/dry ratio

To obtain wet/dry ratios, mice were sacrificed, lungs excised and rinsed briefly in PBS. Lungs were then blotted and weighed to obtain wet weight. Dry weight was taken after incubation of lungs for 3 days at 60°C.

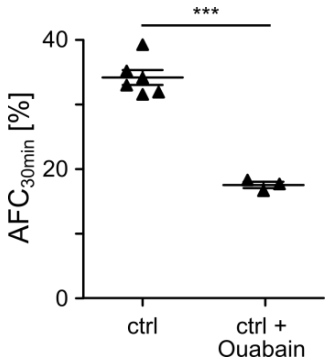
Supplemental Figures and Figure Legends

SI Figure 1.



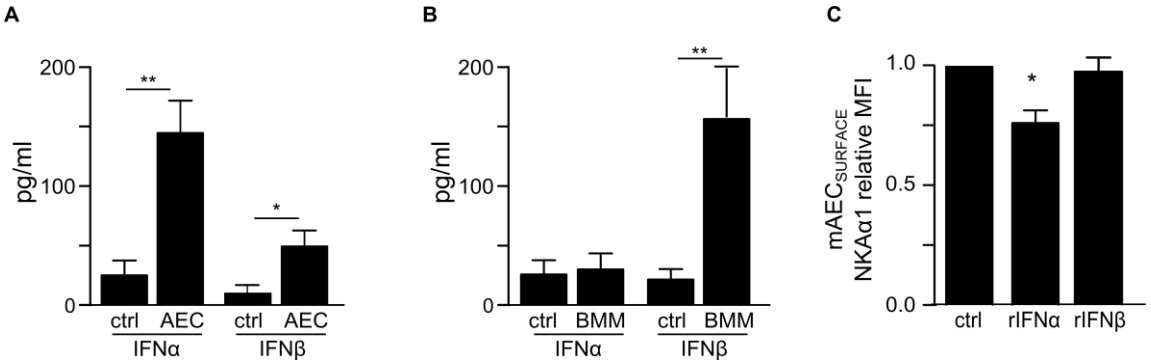
SI Figure 1. Epithelial cells from lung homogenates mainly consist of alveolar epithelial cells. Flow cytometric analysis of the composition of the EpCAM^{positive} cell population from lung homogenate of wt mice after dead cell and doublet exclusion revealed that approximately 95% of cells are of AEC phenotype (composed of 95% AEC II and 5% AEC I) and approximately 5% are integrin α6^{positive} bronchial epithelial cells, as defined by McQualter et al. (2010).

SI Figure 2.



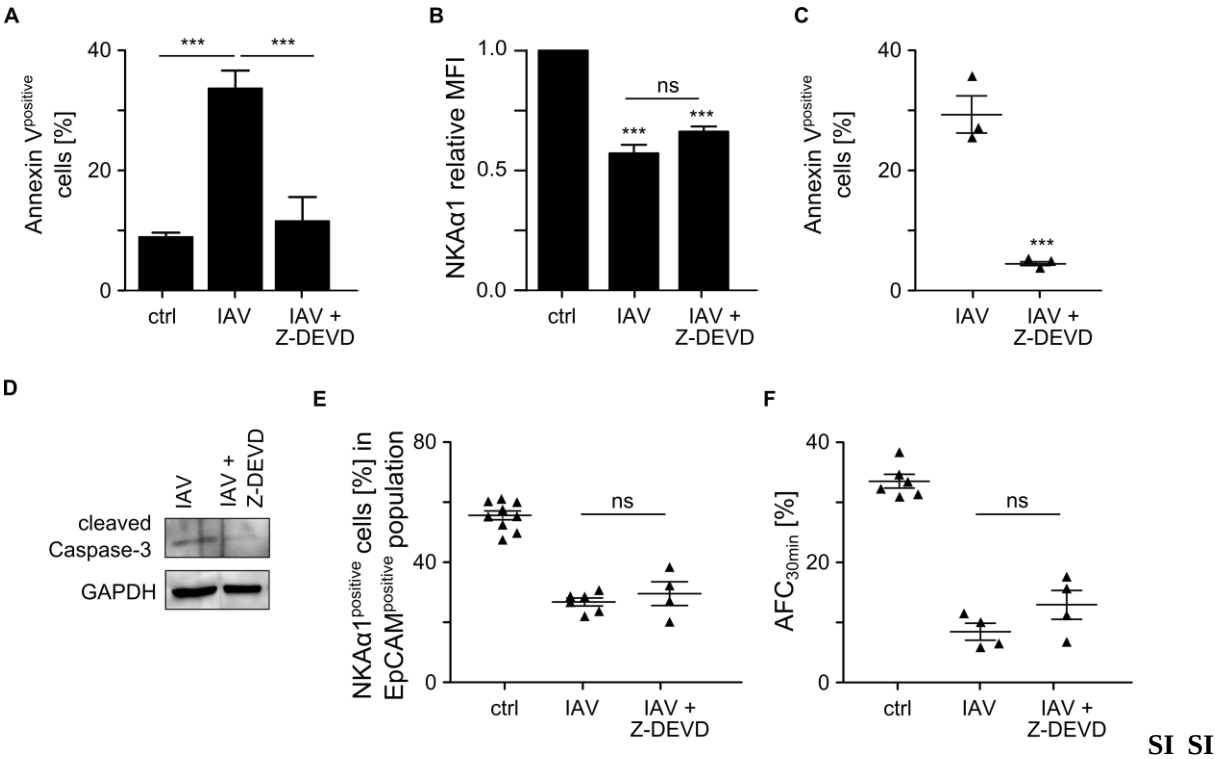
SI Figure 8. Inhibition of Na,K-ATPase activity severly impacts on AFC rates *in vivo*. AFC was measured in uninfected wt mice without further treatment (ctrl) or 30 min after intraperitoneal administration of 3mM Ouabain (ctrl + Ouabain). Graphs show single data points plus means ± SEM of 3-6 mice per group. Statistical significance was analyzed by students's t-test.

SI Figure 3.



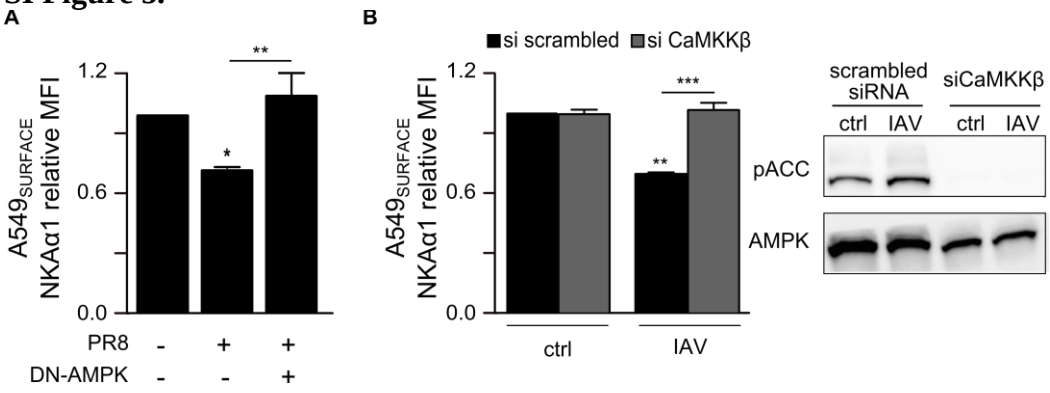
SI Figure 3. Type I IFN induction in AEC upon IAV infection. *(A-B)* IFNα and IFNβ concentrations of supernatant collected 24h after PR8 infection of AEC *(A)* or BMM *(B)* were quantified by ELISA. *(C)* Relative MFI of NKAα1 on mAEC treated with 25U/ml mouse recombinant IFNα (rIFNα) or/and 100pg/ml mouse recombinant IFNβ (rIFNβ) for 16h. Bar graphs represent means ± SEM of 4-6 independent experiments.

SI Figure 4.



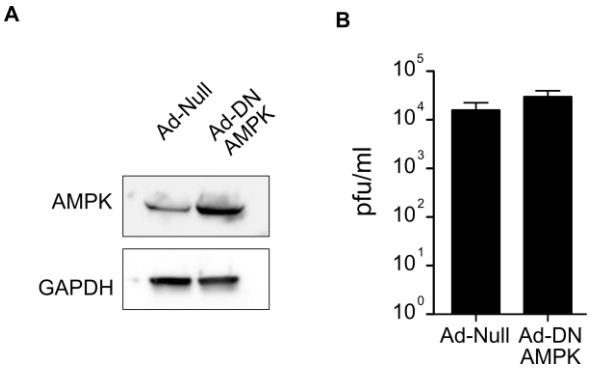
SI SI Figure 4. The TRAIL-mediated reduction of NKAα1 levels is induced independently of epithelial cell apoptosis. *(A, B)* mAEC were inoculated with PBS (ctrl) or PR8 (IAV) and treated with 50µM Z-DEVD (IAV + Z-DEVD) for 24h. *(A)* Apoptosis was quantified by annexin V staining and flow cytometry. *(B)* Relative median fluorescence intensities (MFI) of NKAα1 detected by FACS in mAEC. *(C-F)* Wt mice were inoculated with PBS (ctrl) or PR8 (IAV) and treated with 10mg/kg Z -DEVD s.c at d5 and d6. Mice were sacrificed at 7d pi.*(C)* Apoptosis was quantified by annexin V staining and flow cytometry *(D)* Representative western blot of 3 independent experiments for cleaved caspase-3 and GAPDH from AEC isolated from wt mice inoculated with PBS (ctrl) or PR8 (IAV) and treated with 10mg/kg Z-DEVD s.c at d5 and d6. Mice were sacrificed at 7d pi. *(E)* Flow cytometric analysis of NKAα1 subunit expression on $EpCAM^{positive}$ epithelial cells from lung homogenate. *(E)* In vivo quantification of alveolar fluid clearance (AFC) rates over a time interval of 30 minutes. Representative blots, dot or bar graphs showing means ± SEM of n = 3 experiments for *(A-D)*, n = 4-9 for *(E)* and n = 4-6 for *(F)*. Data set depicting control conditions in *(E, F)* are identical to Figure 1C (AFC) and 3A (NKAα1 expression) and were included for better comparison of between experimental conditions. Statistical significance was analyzed by one-way ANOVA and post-hoc Tukey.

SI Figure 5.



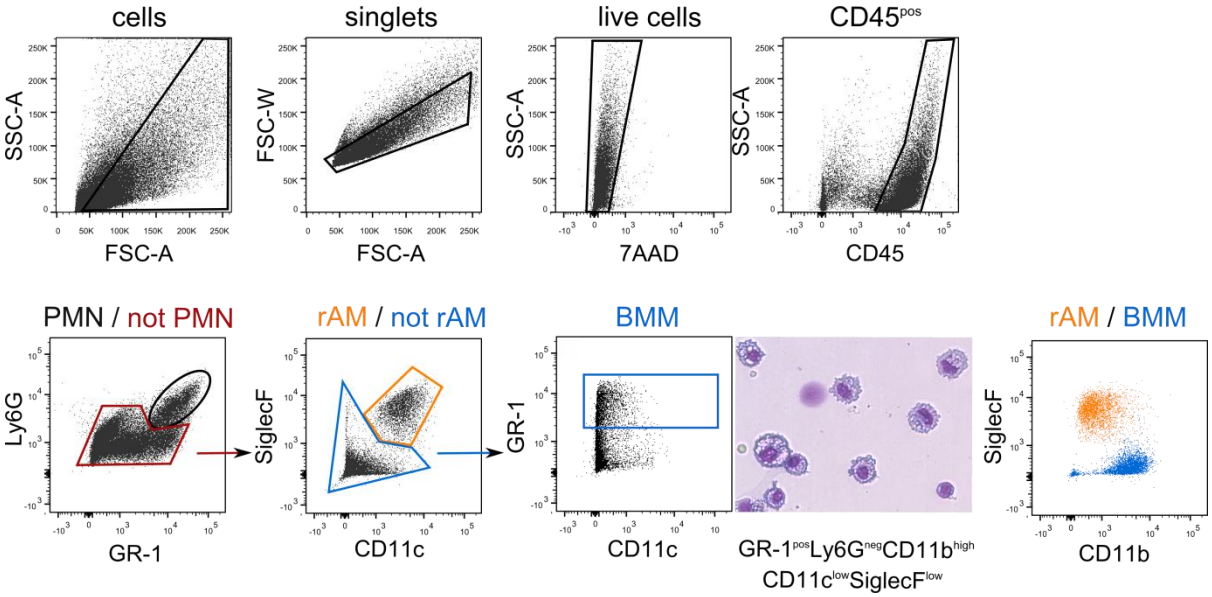
SI Figure 5. Blockade of AMPK and CaMKKβ rescues NKAα1 expression *in vitro*. *(A)* Relative MFI of NKAα1 on A549 cells transduced with DN-AMPK for 24h prior to treatment with PBS (ctrl) or PR8 for additional 24h. Bar graphs represent means ± SEM of 4 independent experiments. *(B)* Left panel shows relative MFI of NKAα1 on A549 cells treated with CaMKKβ-specific siRNA for 48h prior to treatment with PBS (ctrl) or infection with PR8 for additional 24h. Bar graphs represent means ± SEM of 3 independent experiments. Right panel shows representative Western blot of 3 independent experiments of A549 cell lysates for AMPK or its substrate pACC 72h after transfection with scrambled or CaMKKβ-specific siRNA and 24h after PR8 infection.

SI Figure 6.



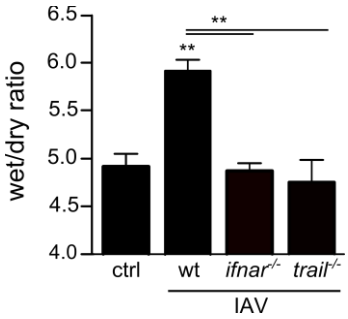
SI Figure 6. Inhibition of AMPK does not influence viral load. *(A)* Representative western blot of 3 independent experiments showing overexpression of dominant-negative AMPK in comparison to house-keeping gene GAPDH in AEC isolated from wt mice inoculated with adenoviral constructs for dominant-negative AMPK (Ad-DN AMPK) or an empty adenoviral construct (Ad-Null). Mice were sacrificed at d5 after adenoviral delivery. *(B)* Viral titers from lavage of wt mice inoculated with IAV (Udorn) at d3 after i.t. infection with adenovirus overexpressing NKAα1 and. Mice were sacrificed at 2d after IAV infection. *(C)* Mice were inoculated with PR8 (IAV) with or without treatment with 20mg/kg Compound C i.p. at d5 pi and sacrificed at d7 pi. Infected (HA^{pos}) versus non-infected (HA^{neg}) cell populations of isolated distal epithelial cells were analyzed by flow cytometry. Bar graphs represent means ± SEM of 4 mice per group. Statistical significance was analyzed by one-way ANOVA and post-hoc Tukey.

SI Figure 7.



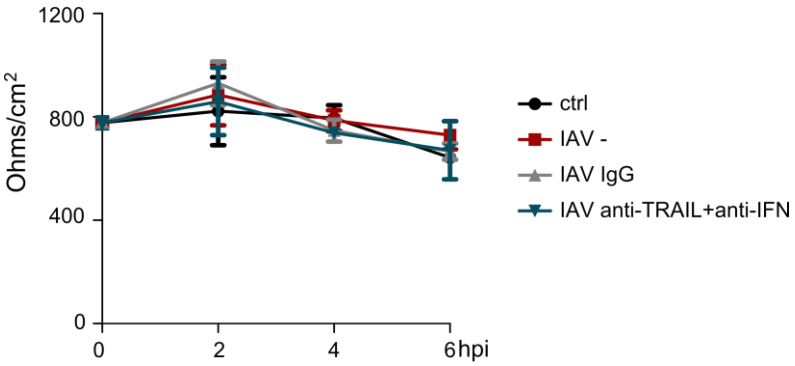
SI Figure 7. Flow-sorting strategy of BMM derived from bronchoalveolar lavage of IAV-infected mice. Gating strategy showing representative dot plots for identification of BMM after doublet exclusion and live/dead staining by the following marker expression: $CD45^{pos}$ $GR\text{-}1^{pos}$ $Ly6G^{neg}$ $CD11b^{high}$ $CD11c^{low}$ $SiglecF^{low}$. BMM were obtained by bronchoalveolar lavage from mice at d7 pi with 500pfu PR8. Purity of sorted BMM was determined by flow cytometry as well as Pappenheim-stained cytospins.

SI Figure 8.



SI Figure 8. *Ifnar*$^{-/-}$ and *trail*$^{-/-}$ mice are protected from edema formation. Lung wet/dry ratios were obtained from wt, *ifnar*$^{-/-}$ or *trail*$^{-/-}$ mice inoculated with PBS (ctrl) or PR8 (IAV) and sacrificed 7d pi.

SI Figure 9.



SI Figure 9. Human AEC co-cultured with human primary AM retain barrier integrity after IAV infection. hAEC were cultured in transwell cell culture vessels for approximately 10 days and monitored for establishment of an electrochemical resistant monolayer by trans-endothelial resistance (TER) measurements. Cultures displaying at least resistances of ≥ 600 Ω/cm^2 were then co-cultured together with bottom-well seeded primary isolated human alveolar macrophages (purity ≥ 92%) and inoculated with PBS (ctrl) or PR8 at MOI 1 (IAV). Additionally to IAV infection, cultures were left untreated (IAV -) or supplemented with isotype control antibodies (IAV + IgG) or neutralizing antibodies against human TRAIL (0.1µg/ml) and human IFNα 0.5µg/ml). TER was measured every at the indicated time points after infection. Data points represent means ± SEM of 4 independent experiments.

SI Table 1.

	D0	D1	D2	D3	D4	D5	D6	D7
IgG+BSA	0 (±0)	0 (±0)	0 (±0)	0 (±0)	0 (±0)	5 (±0)	5 (±0)	13 (±1.23)
anti-TRAIL +anti-IFNα	0 (±0)	0 (±0)	0 (±0)	0 (±0)	0 (±0)	0 (±0)***	1 (±1)**	4 (±2.92)***

Table 1. Therapeutic application of anti-TRAIL and anti-IFNα antibodies improves morbidtity scores after IAV-infection. Wt mice were inoculated with 500pfu PR8 (IAV) and treated with i.p. administration of 150µg IgG control antibody or anti-TRAIL antibody at d3 and d5 pi together with a BSA vehicle control or anti-IFNα antibody (10.000IU) i.t. at d3 pi (IgG+BSA or anti-TRAIL+anti-IFNα, respectively) and sacrificed at d7 pi. Mice were scored daily according to the following clinical scoring system: 5 points each for either opaque eyes/enophthalmus, ruffled fur, reduced activity, or more frequent and laborious breathing; 10 points for body weight loss of more than 10% within two days; 15 points for body weight loss of more than 20% within two days. Table represents daily morbidity score means ± SEM of 5 mice per group. Statistical significance was analyzed by one-way ANOVA and post-hoc Tukey.