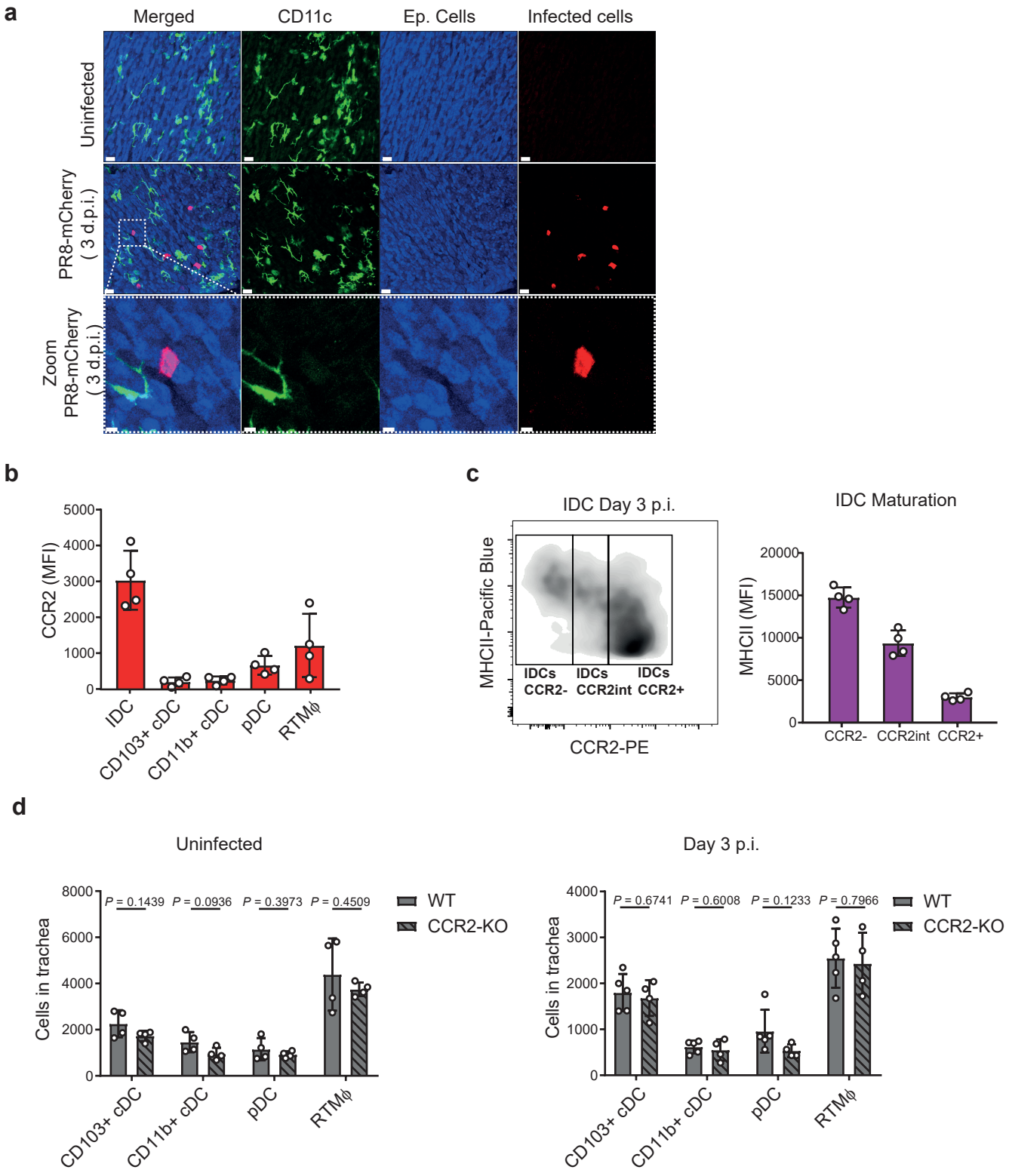
Protection against influenza infection requires early recognition by inflammatory dendritic cells through C-type lectin receptor SIGN-R1

Miguel Palomino-Segura, Laurent Perez, Yagmur Farsakoglu, Tommaso Virgilio, Irene Latino, Rocco D'Antuono, Nikolaos Chatziandreou, Diego U. Pizzagalli, Guojun Wang, Adolfo García-Sastre, Federica Sallusto, Michael C. Carroll, Olivier Neyrolles, and Santiago F. Gonzalez.

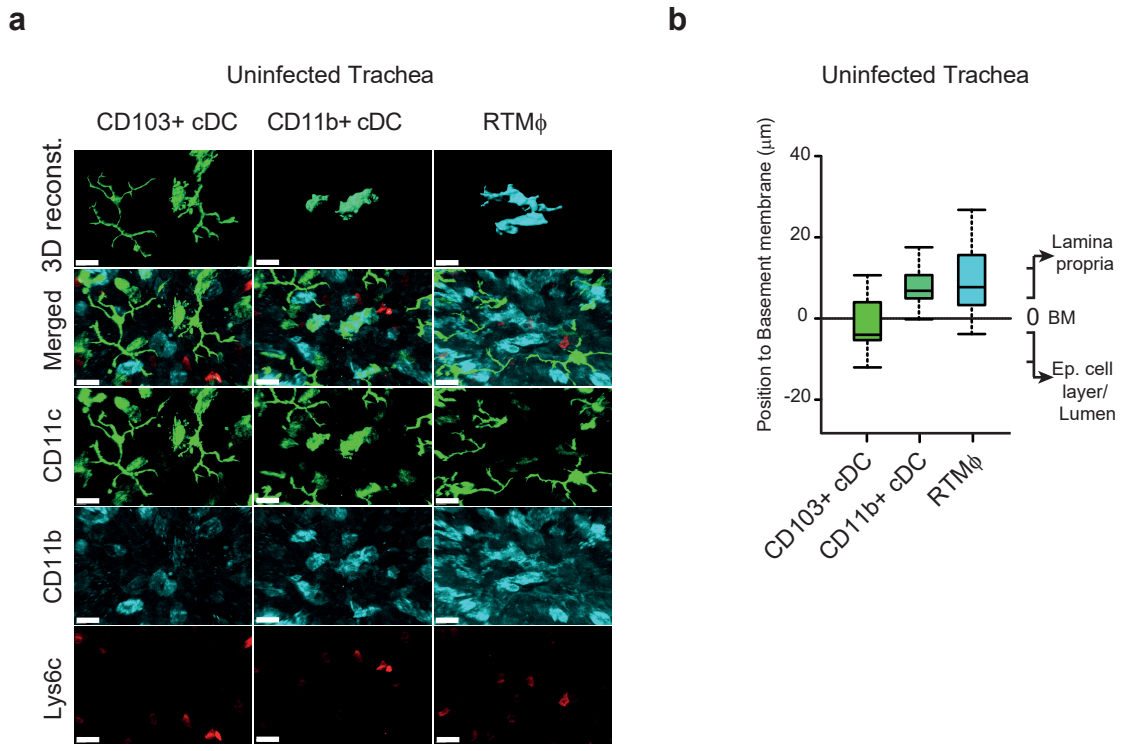
Supplementary information

Supplementary Figures1-6

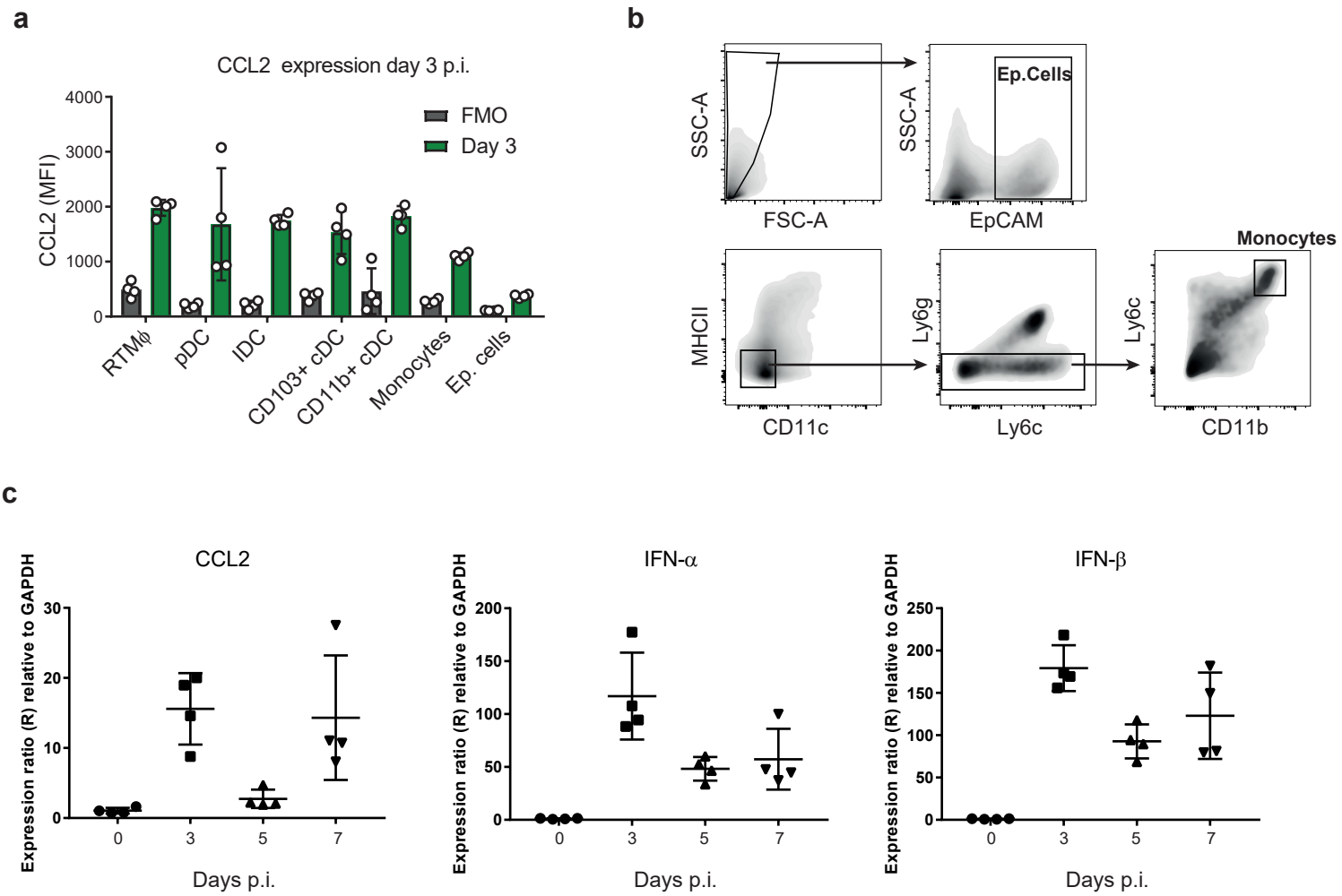
Supplementary Table 1 and 2



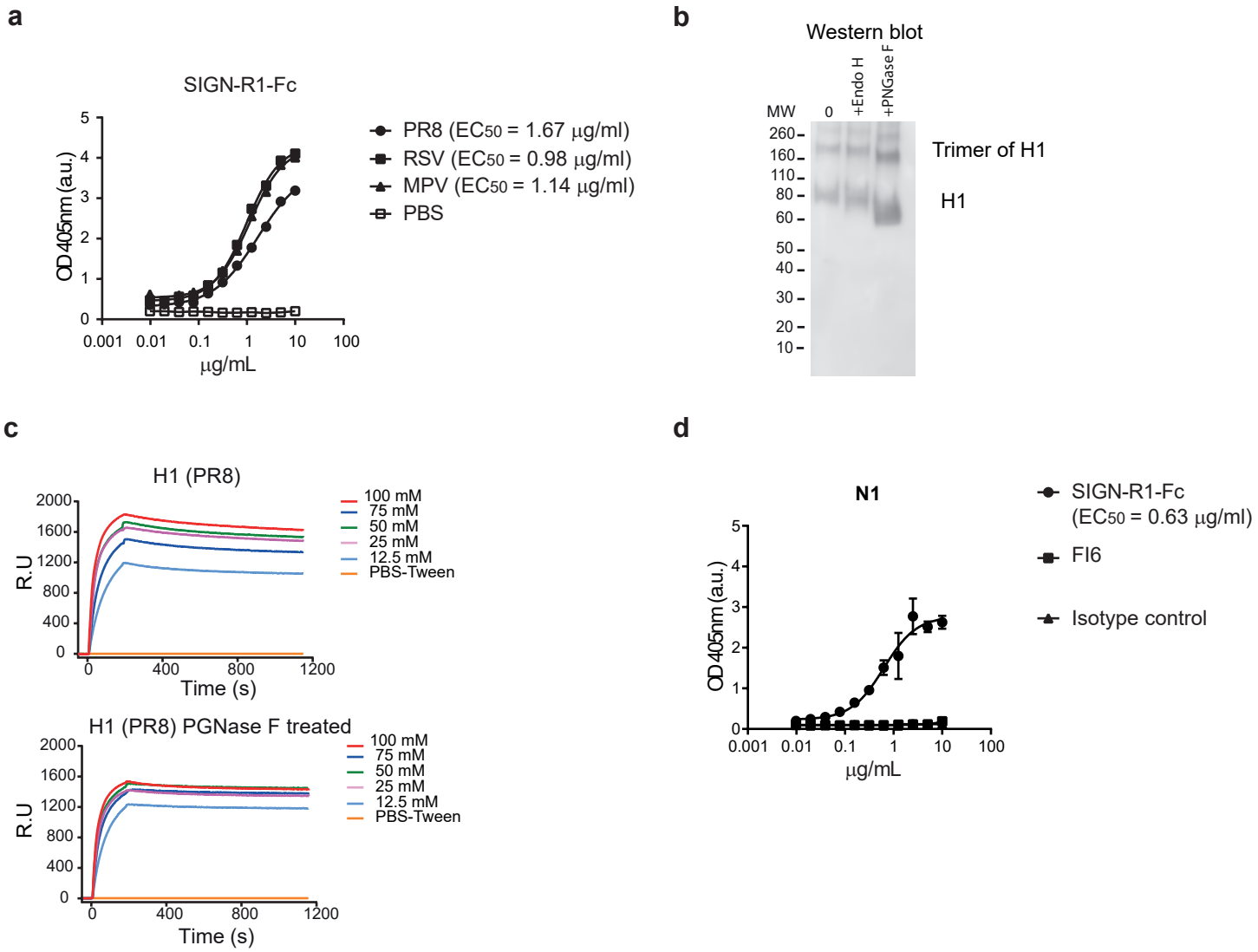
Supplementary Figure 1. a, Confocal micrographs from tracheal explants showing the network of CD11c+ cells (green) and the epithelial cells (blue) from uninfected and infected mice at day 3 p.i.. Mice were infected with a recombinant influenza virus that carried a mCherry-reporter gene in the NS segment (PR8-mCherry), which allowed the visualisation of infected cells (red). The scale bar represents 20µm in the first two rows and 5µm in the third row (magnified panels). **b**, Flow cytometry data showing expression levels of CCR2 in subsets of tracheal DC and RTMφ (n = 4 mice). **c**, Representative scatterplot (left) and quantification graph (right) of mouse tracheal IDC at day 3 p.i. showing how the loss of CCR2 expression correlates with the upregulation of the maturation marker MHCII. According to this, IDC were divided in three populations (CCR2-, CCR2 int, CCR2+) based on their CCR2 expression (n = 4 mice). **d**, Flow cytometric analysis of tracheal DC and RTMφ from WT and CCR2-KO in uninfected conditions (left) and at day 3 p.i. (right) (n = 4 and 5 mice per respective time). The presented data are representative of at least three independent experiments. Results are given as mean ± SD. Data were analysed using the two-tailed Student's t-test.



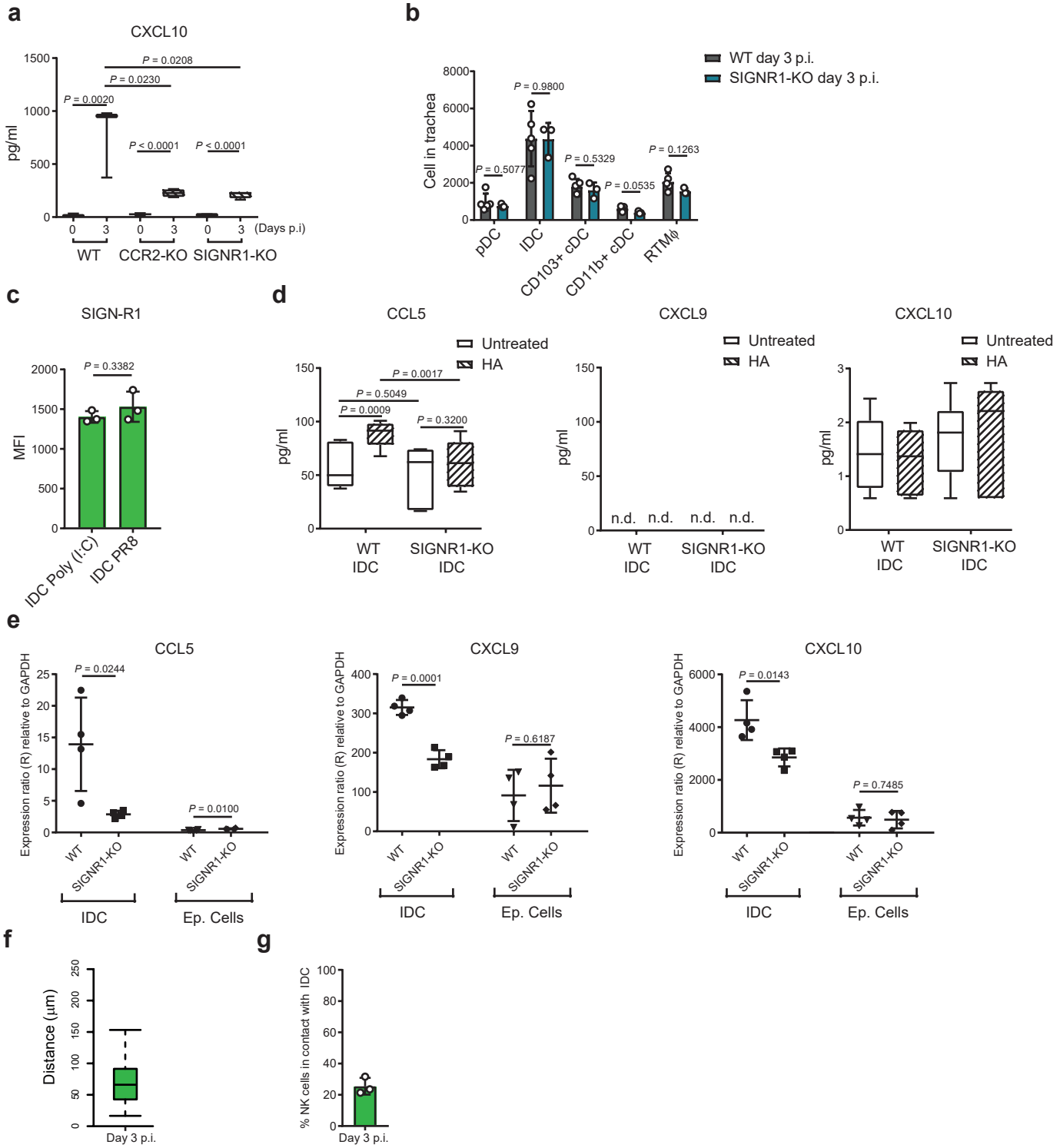
Supplementary Figure 2. a, Representative confocal micrographs showing the 3D reconstruction of the DC subsets and RTMφ, located in the tracheal mucosa of a WT animal. Cells are characterised based on the expression of CD11c (green), CD11b (light blue) and Ly6c (red). CD103+ cDC are defined as: CD11c+ / CD11b- / Ly6c-; CD11b+ cDC: CD11c+ / CD11b+ / Ly6c-; and RTMφ CD11c- / CD11b+ /Ly6c-. Scale bar represents 20 µm. **b**, Box plot representing the position of the different group of cells with respect to the basement membrane in the tracheal mucosa of a control animal. The value of the position of the basement membrane was considered 0, cells located towards the lamina propria received positive values while cells located towards the luminal side received negative values. (data are based in the analysis of 3 confocal reconstructions; IDC, n = n.d. ; CD103+ cDC, n = 58; CD11b+ cDC, n = 70; RTMϕ, n =75). The presented data are representative of at least three independent experiments. In **b**, the box plots show 25th to 75th percentiles and median, and the whiskers show 5th to 95th percentiles.



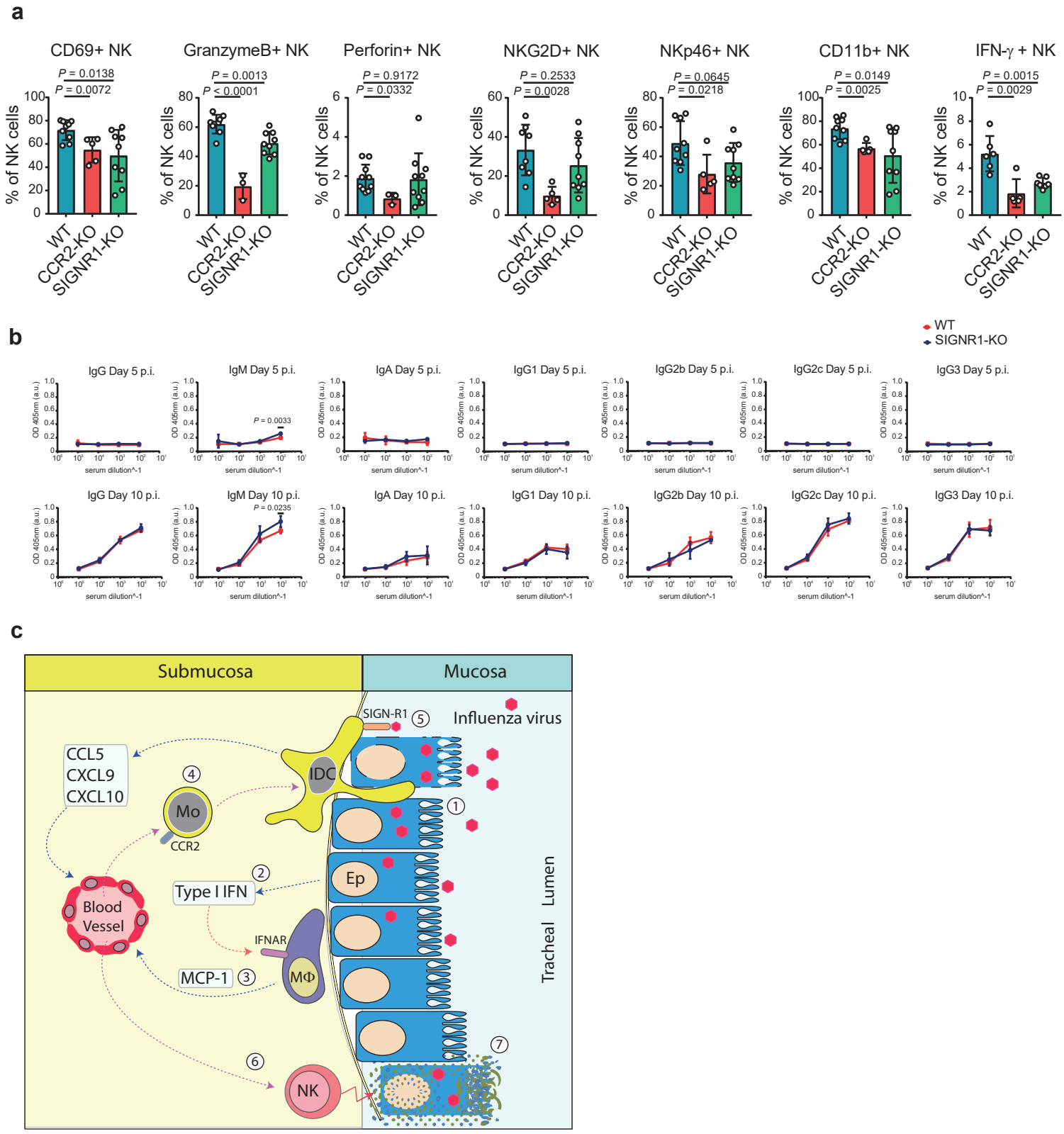
Supplementary Figure 3. a, Flow cytometric analysis showing the intracellular expression levels of CCL2 in tracheal cells at day 3 p.i. compared to the fluorescence-minus-one (FMO) control (n = 4 mice per group). **b**, Flow cytometric characterisation of epithelial cells (EpCAM+) and monocytes (CD45+ / MHCII- / CD11c- / CD11b+ / Ly6chi / Ly6g-). **c**,Time course showing RNA expression levels of the cytokines CCL2, IFN-α and IFN-β in tracheas from PR8 infected mice at 0, 3, 5 and 7 days p.i. (n = 4 mice per group). The presented data are representative of at least three independent experiments. Results are given as mean ± SD.



Supplementary Figure 4. SIGN-R1 interacts with strong affinity with N-linked glycans. a, ELISA analysis of SIGN-R1-Fc binding to PR8, RSV and MPV. The absorbance at 405 nm is plotted against increasing concentrations of recombinant SIGN-R1-Fc (n = 2 replicates per group). **b**, Western blot analysis of deglycosylated H1 by PNGase F glycosidase. After 1 h of treatment H1 is complitely deglycosylated by PNGase F and migrate at a lower molecular weight in a reducing SDS-PAGE. **c**, SPR analysis of untreated and PGNase F-treated H1 reveal no change in affinity when H36-7 mAb is used, indicating that H1 is correctly folded. **d**, ELISA analysis of SIGN-R1-Fc binding to N1.The absorbance at 405nm is plotted against the concentration of antibody added to wells with the same concentartion of recombinant N1. (SIGN-R1, n = 3; FI6, n = 3; Isotype control, n = 2 replicates per group). The presented data are representative of at least of two independent experiments (**a**-**d**). Results are given as mean ± SD (**a**) or mean alone (**d**).



Supplementary Figure 5. a, Levels of secreted CXCL10 in trachea from CCR2-KO, SIGNR1-KO and WT mice at 0 and 3 days p.i. . (WT, n = 5 and 3; CCR2-KO, n = 4 and 4; SIGNR1-KO, n = 4 and 4 mice per respective time point). **b**, Flow cytometric analysis of tracheal DC and RTMφ from WT and SIGNR1-KO at day 3 p.i. . (WT, n = 5; SIGNR1-KO, n = 3 mice per group). **c**, Flow cytometric analysis showing the expression levels of SIGN-R1 in IDC generated by intranasal administration of 40 μg/mice of Poly (I:C) and in tracheal IDC at day 3 p.i. (n = 3 mice per group). **d**, Cytokine levels of secreted CCL5, CXCL9 and CXCL10 by Poly (I:C)-generated IDC from WT and SIGNR1-KO mice after 12 hours of *in vitro* culture with Inflexal® V (WT, n = 8; SIGNR1-KO, n = 10 mice per group). **e**, RNA expression levels of the CCL5, CXCL9 and CXCL10 in sorted IDC and epithelial cells from infected WT and SIGNR1-KO mice at day 3 p.i. (n = 4 mice per group). **f**, Box plot representing the distance between tracheal NK cells and the nearest neighbour cell in the IDC population at day 3 p.i. (data are based on the analysis of 3 confocal reconstructions; NK cells, n = 102). **g**, Frequency of tracheal NK cells that are in contact with an IDC at day 3 p.i.. Contact between cells was considered when their distance (closest voxels) is less than or equal to 5 μm (data are based on the analysis of 3 confocal reconstructions). The presented data are representative of at least three independent experiments (**a**-**c** and **e**-**g**) or at least two independent pooled experiments (**e**). In **a**, **d** and **f**, the box plots show 25th to 75th percentiles and median, and the whiskers show minimum and maximum values (**a**, **d**) or 5th to 95th percentiles (**f**). In **b**, **c**, **e**, and **g**, results are given as mean ± SD. Data were analysed using the two-tailed Student's t-test.



Supplementary Figure 7. a, Flow cytometric quantification of frequency of CD69+, granzymeB+, perforin+, NKG2D+, NKp46+, CD11b+ and IFN-γ+ NK cells in WT, SIGNR1-KO and CCR2-KO animals at day 3 p.i. (WT, n = 9, 9, 10, 8, 9, 9 and 6; CCR2-KO, n = 5, 3, 3, 5, 5, 5, and 5; SIGNR1-KO, n = 9, 9, 10, 9, 9, 9, and 8 mice per respective marker). **b**, ELISA of serum collected from WT and SIGNR1-KO mice at day 5 and 10 p.i. indicating the absorbance of PR8-specific antibodies detected in serial dilutions of serum (n = 4 per group). **c**, Proposed working model. Epithelial cells (Ep) get infected in the lumen of the trachea (1) and initiate the Type I IFN response (2), which in turns induce the activation of macrophages (MΦ) and the production MCP-1 (3). The release of this chemokine in the bloodstream induces the recruitment of CCR2 expressing monocytes (Mo) (4), which then differentiate into inflammatory dendritic cells (IDC) and relocate towards the luminal part of the trachea. The majority of the IDC express the C-type lectin receptor SIGN-R1 that mediates the recognition of the virus (5), the activation of the IDC and regulated the production of the chemokine CCL5, CXCL9 and CXCL10. The latter induced the recruitment of NK cells (6), which control the replication of the virus in the epithelium (7). The presented data are representative of at least two independent pooled experiments (**a**) or at least three independent experiments (**b**). In **a** and **b**, results are given as mean ± SD. Data were analysed using the two-tailed Student's t-test.

Supplementary Table 1. List of antibodies.

Antibody target	Clone	Color	Manufacturer
αLy-6C	HK1.4	PE, Biotin	Biolegend
αF4/80	BM8	APC/Cy7, AF488	Biolegend
αB220	RA3-6B2	AF647, PE/Cy7, BV605	Biolegend
αCD103	2E7	AF647, AF488	Biolegend
αCD11b	M1/70	AF488, BV785, AF647	Biolegend
αI-A/I-E (MHCII)	M5/114.15.2	Pacific Blue, BV650	Biolegend
αEpCAM	G8.8	PE/Cy7	eBioscience
αCD45	30-F11	AF700	Biolegend
αCD45.2	104	PE/Cy7	Biolegend
αCD11c	N418	AF647, APC/Cy7, Bv711	Biolegend
αIFN-β	RMMB-1	FITC	PBL assay science
αCCL2	2H5	Pacific Blue*	BioXcell
αSIGN-R1	22D1	CF405M**, Atto647***	BioXcell
Armenian hamster IgG	(αSIGN-R1 isotype control)	Pure	Bioxcell
αCCL5	2E9/CCL5	PE	Biolegend
αCXCL9	MIG-2F5.5	AF647	Biolegend
αCD3ε	eBio500A2	eFluor450	eBioscience
αNK1.1	PK136	AF488, PerCP-Cy5.5	Biolegend, eBioscience
αCCR5	HM-CCR5	PE	Biolegend
αCXCR3	CXCR3-173	PE	Biolegend
αCCR2	475301	PE	R&D Systems

αCD69	H1.2F3	FITC	Biolegend
αGranzymeB	GB11	AF647	Biolegend
αPerforin	eBioOMAK-D	PE	eBioscience
αNKG2D	CX5	APC	Biolegend
αNKp46	29A1.4	eFluor450	eBioscience
αIFN-γ	XMG1.2	BV785	Biolegend
αLy-6G	1A8	BV711	Biolegend
αCD16/32	93	Pure	Biolegend
αGr-1	RB6-8C5	AF488	Biolegend
αCD31	390	AF647	Biolegend

** In-house labelled with Pacific Blue™ Antibody Labelling Kit (ThermoFisher Scientific),** In-house labelled with CF405M Mix-n-Stain™ Antibody Labelling Kit (Biotium), *** In-house labelled with Atto 647 NHS ester (Atto-Tec).*

Supplementary Table 2. List of primers.

Accession number	Gene	Forward Primer (Direction 5'-3')	Reverse Primer (Direction 5'-3')
111654	*Ifnα*	CTGCTGGCTGTGAGGA	CACCTCCCAGGCACA
15977	*Ifnβ*	TGTCCTCAACTGCTCTCCAC	AAGATCTCTGCTCGGACCAC
20296	*Ccl2*	CTCTTCCTCCACCACCATGC	GGTGAATGAGTAGCAGCAGGT
16173	*Il18*	GGTTCCATGCTTTCTGGACT	GGCCAAGAGGAAGTGATTTG
20304	*Ccl5*	GCCAACCCAGAGAAGAAGTG	AGCAAGCAATGACAGGGAAG
17329	*Cxcl9*	CTTTTGGGCATCATCTTCCT	TTGAGGTCTTTGAGGGATTTG
15945	*Cxcl10*	CGTCATTTTCTGCCTCATCC	TTCCCTATGGCCCTCATTCT
14433	*GAPDH*	ACATCATCCCTGCATCCACT	AGATCCACGACGGACACATT