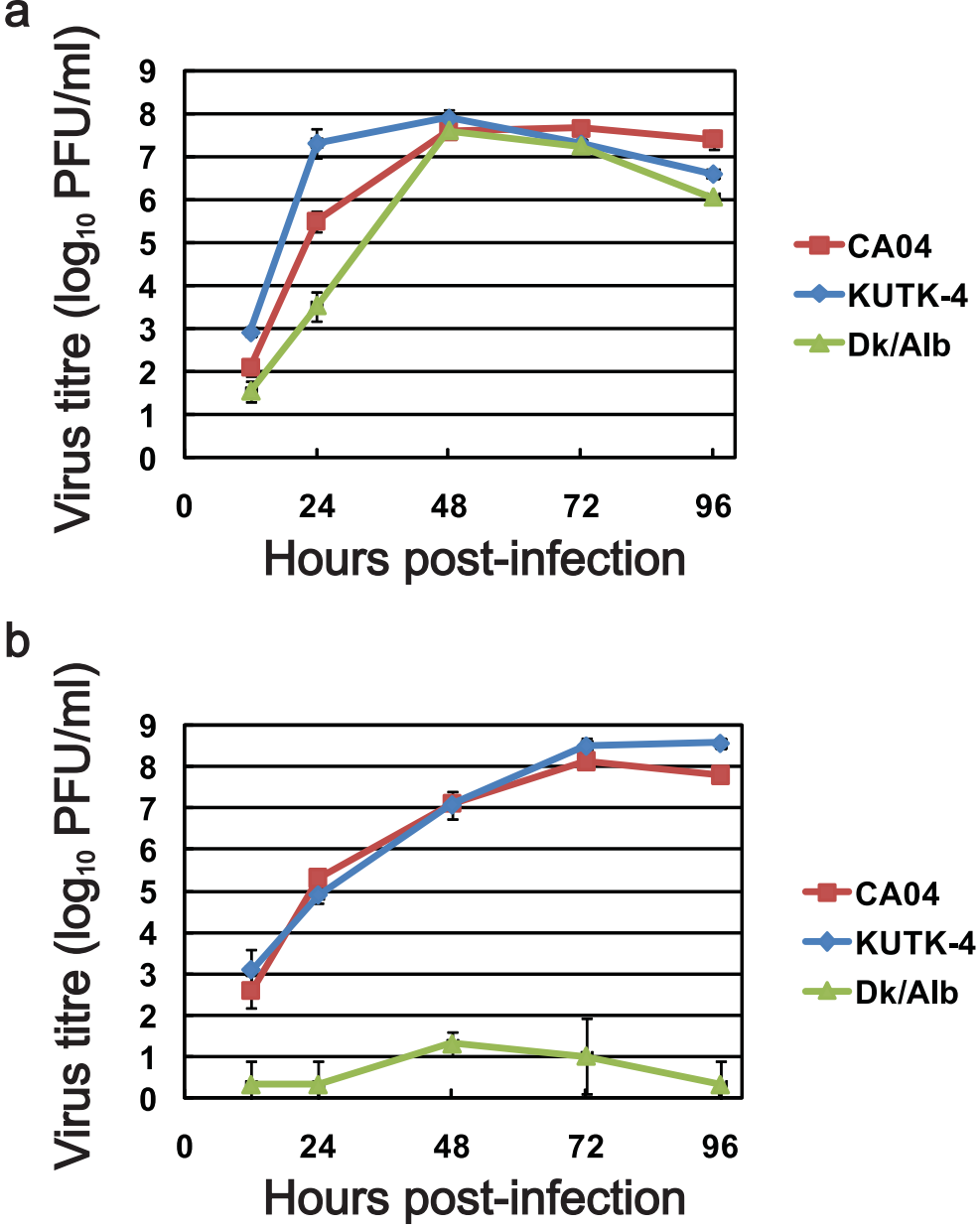
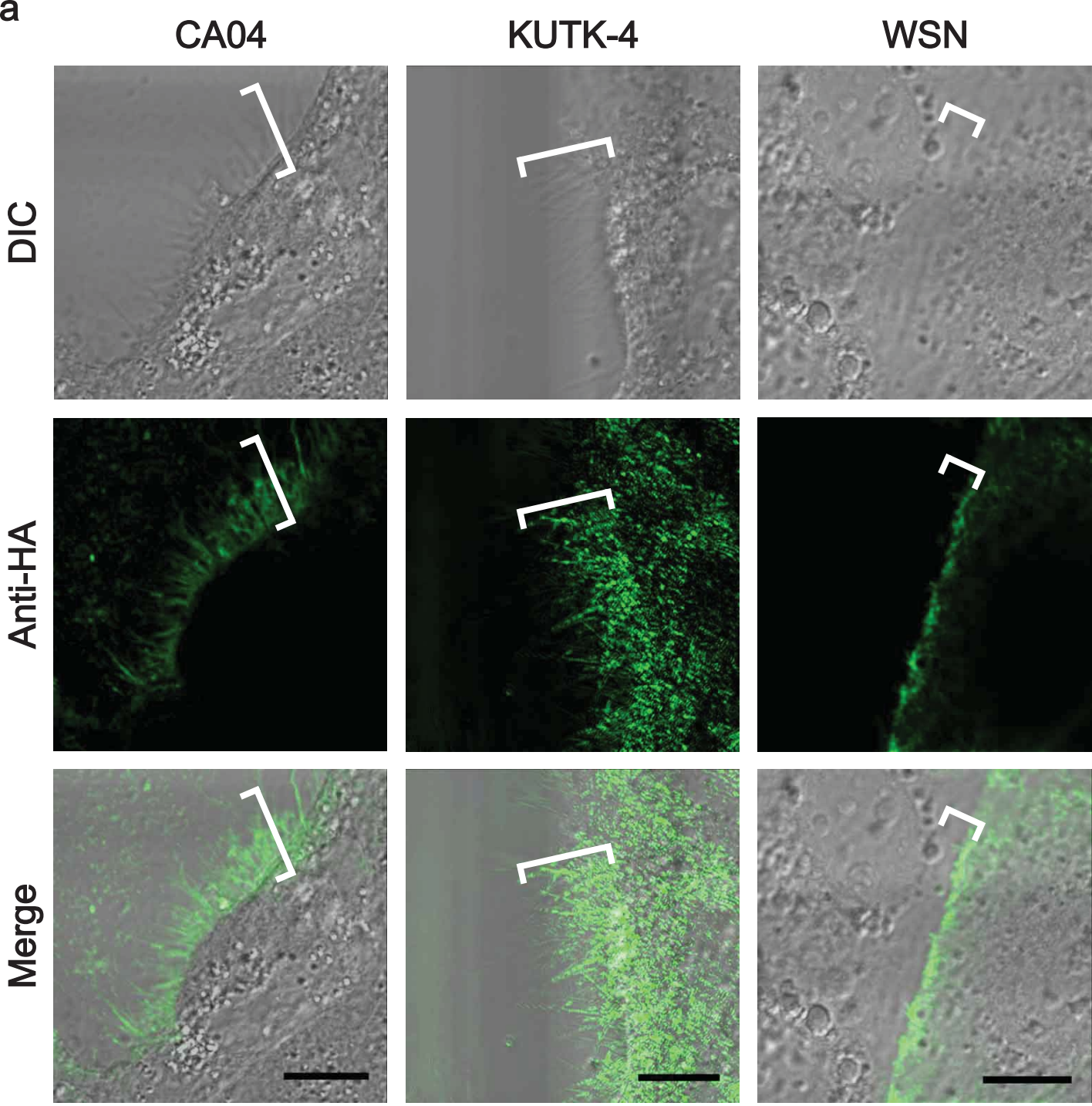
Supplementary Information

- Supplementary figures

- Supplementary tables

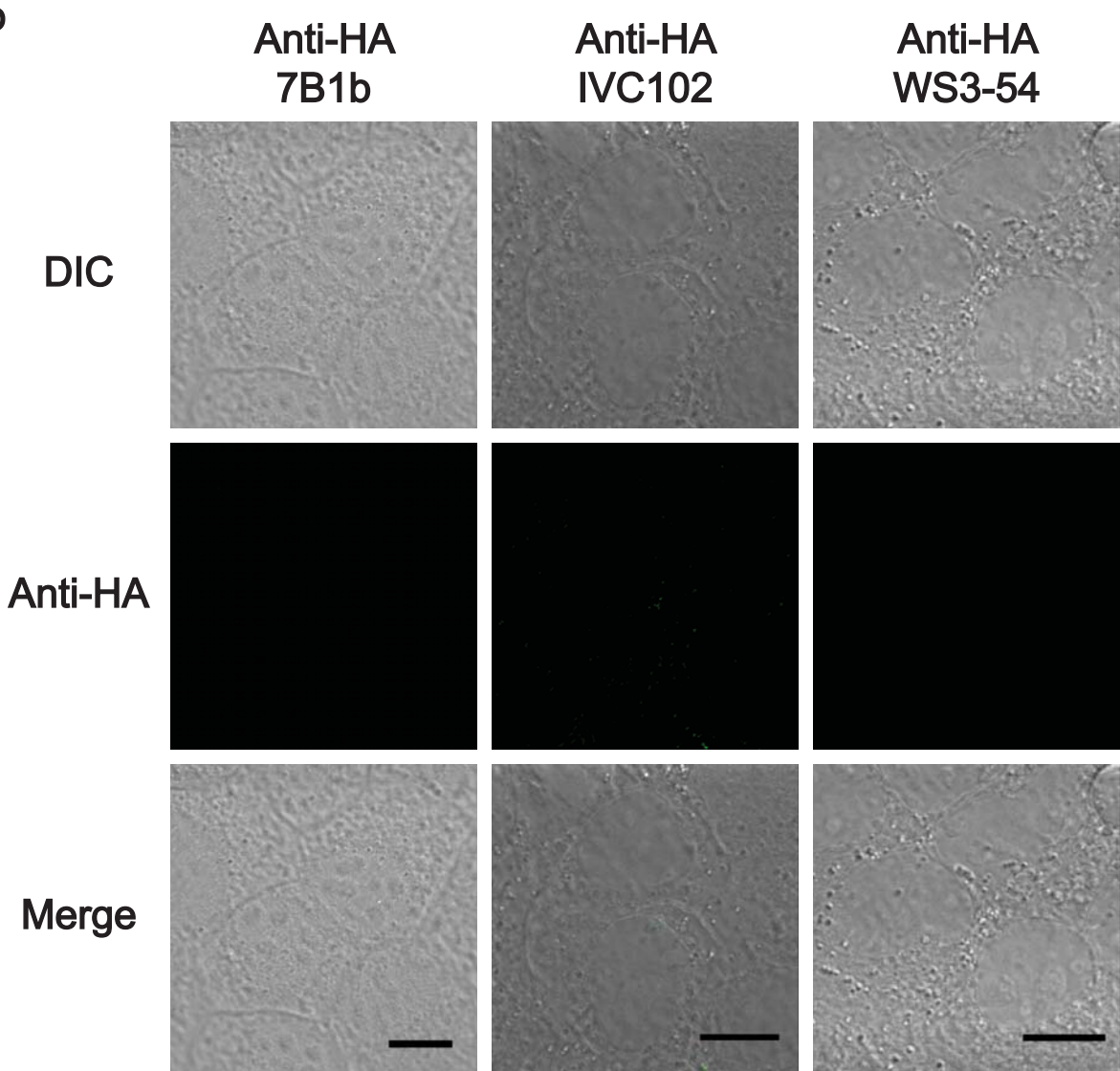


Supplementary Figure S1. Growth properties of viruses in cells. MDCK cells were infected with CA04 (red), KUTK-4 (blue), or A/duck/Alberta/35/76 (H1N1; Dk/Alb, green) at an MOI of 0.001 (**a**). Differentiated human airway epithelial cells were infected with CA04 (red), KUTK-4 (blue), or Dk/Alb (green) at an MOI of 0.001 (**b**). The supernatants of infected cells were harvested at the indicated times and virus titres were determined by plaque assays in MDCK cells. Error bars indicate standard deviations from three independent experiments.



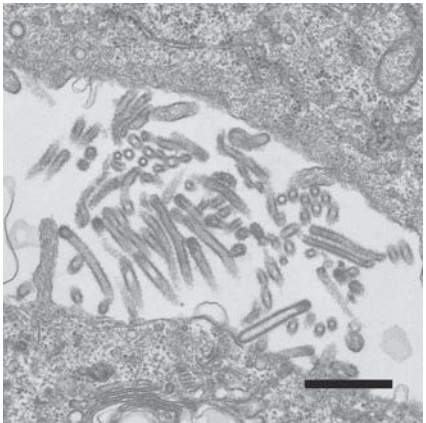
Supplementary Figure S2. Morphology of budding CA04 virions. MDCK cells infected with A/California/04/09 (H1N1) (CA04, left), A/Kawasaki/UTK-4/09 (H1N1) (KUTK-4, middle), or A/WSN/33 (H1N1) (WSN, right) were examined with confocal microscopy (**a**); mock-infected cells were processed similarly (**b**). Virus-infected cells were observed by transmission electron (TEM, **c**) and scanning electron microscope (SEM, **d**)[2]. Brackets in **a** and arrows in **d** indicate budding viruses. Scale bar: **a and b**, 10 μm; **c**, 500 nm; **d**, 5 (upper panels) and 2 μm (lower panels). DIC, differential interference contrast.

b

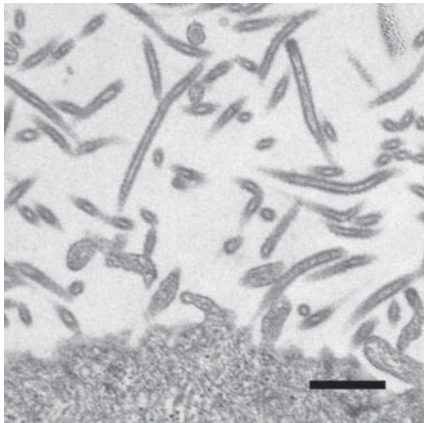


C

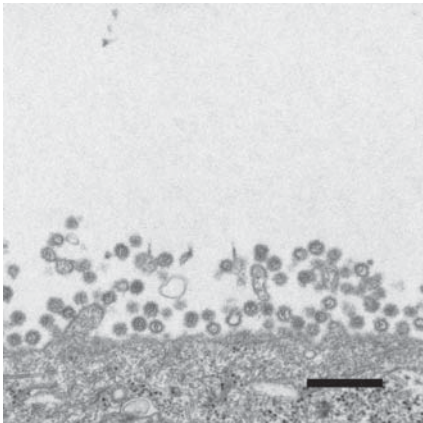
CA04



KUTK-4



WSN

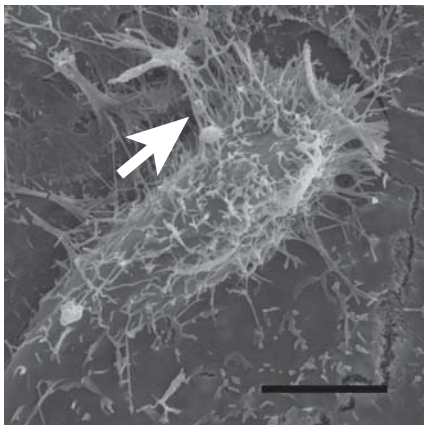


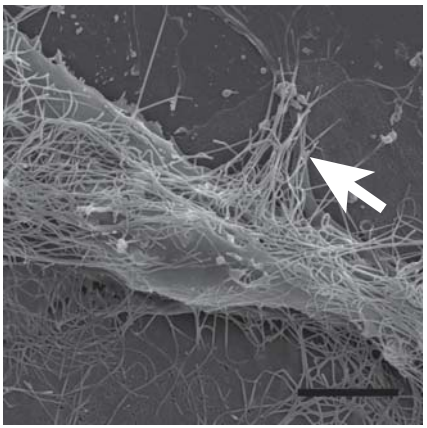
d

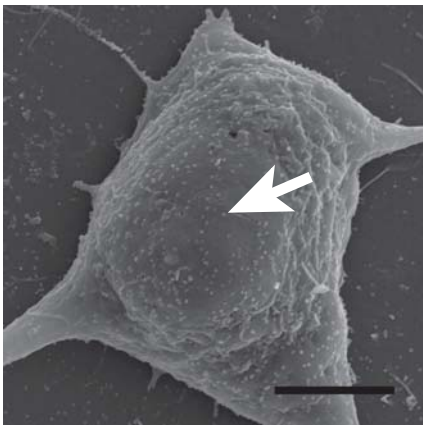
CA04

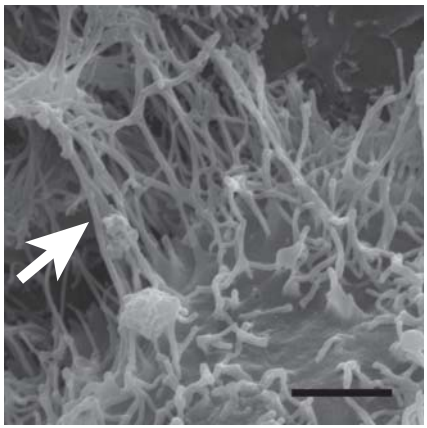
KUTK-4

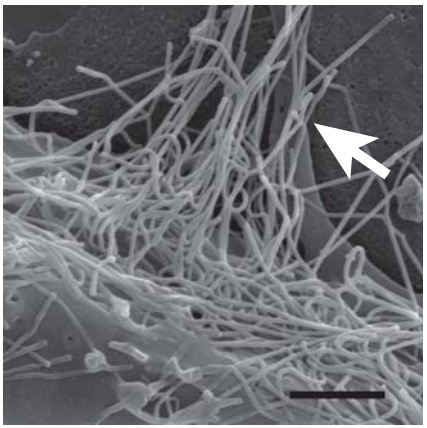
WSN

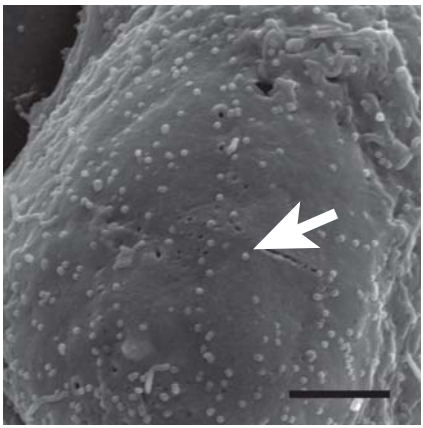




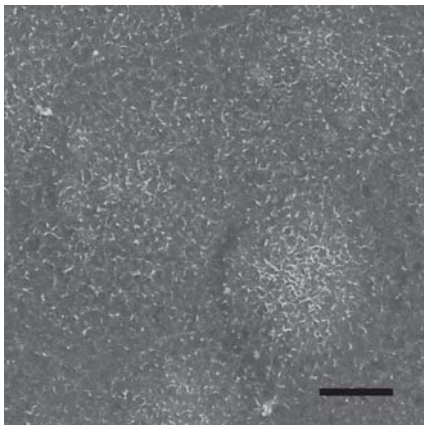


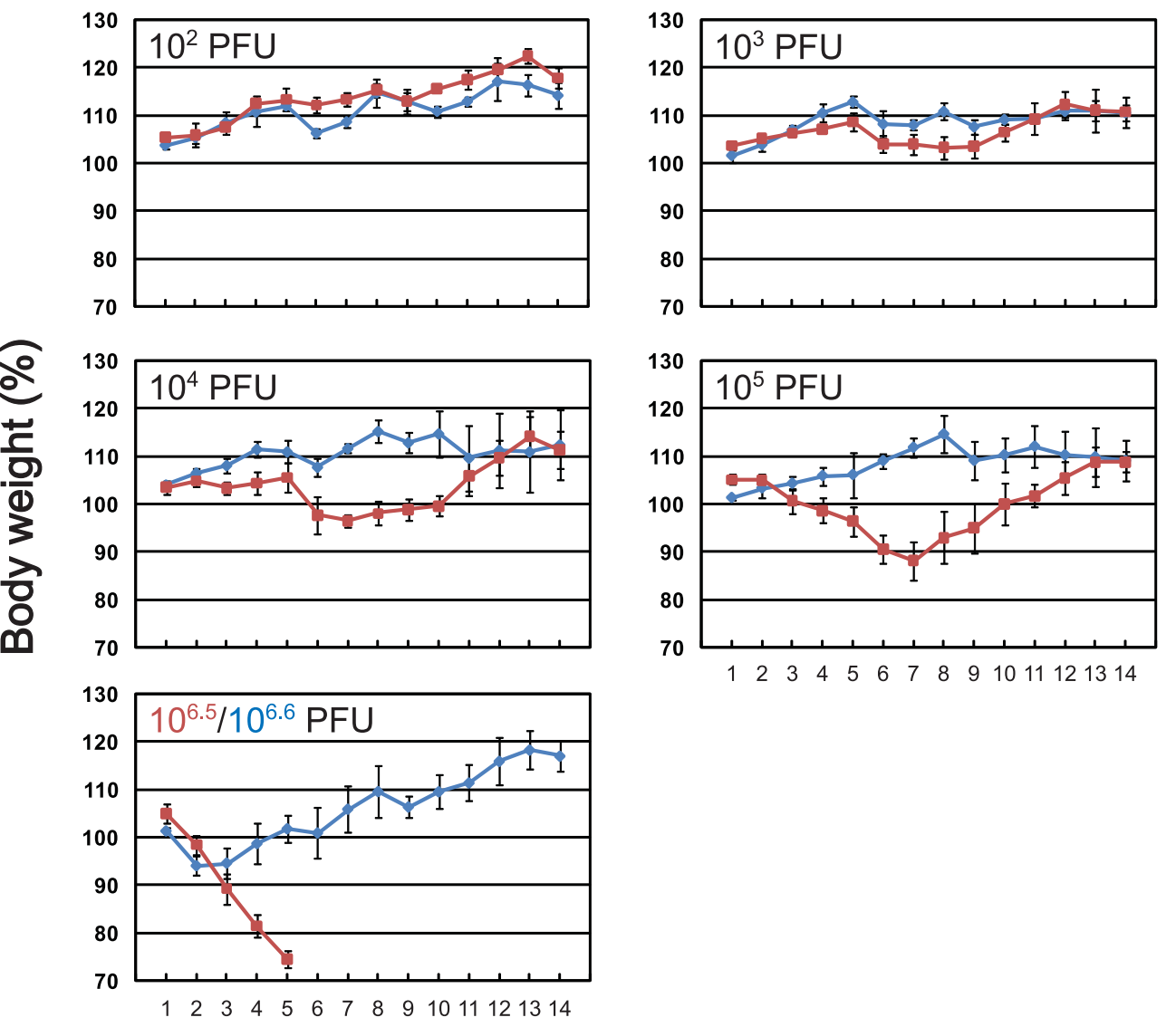




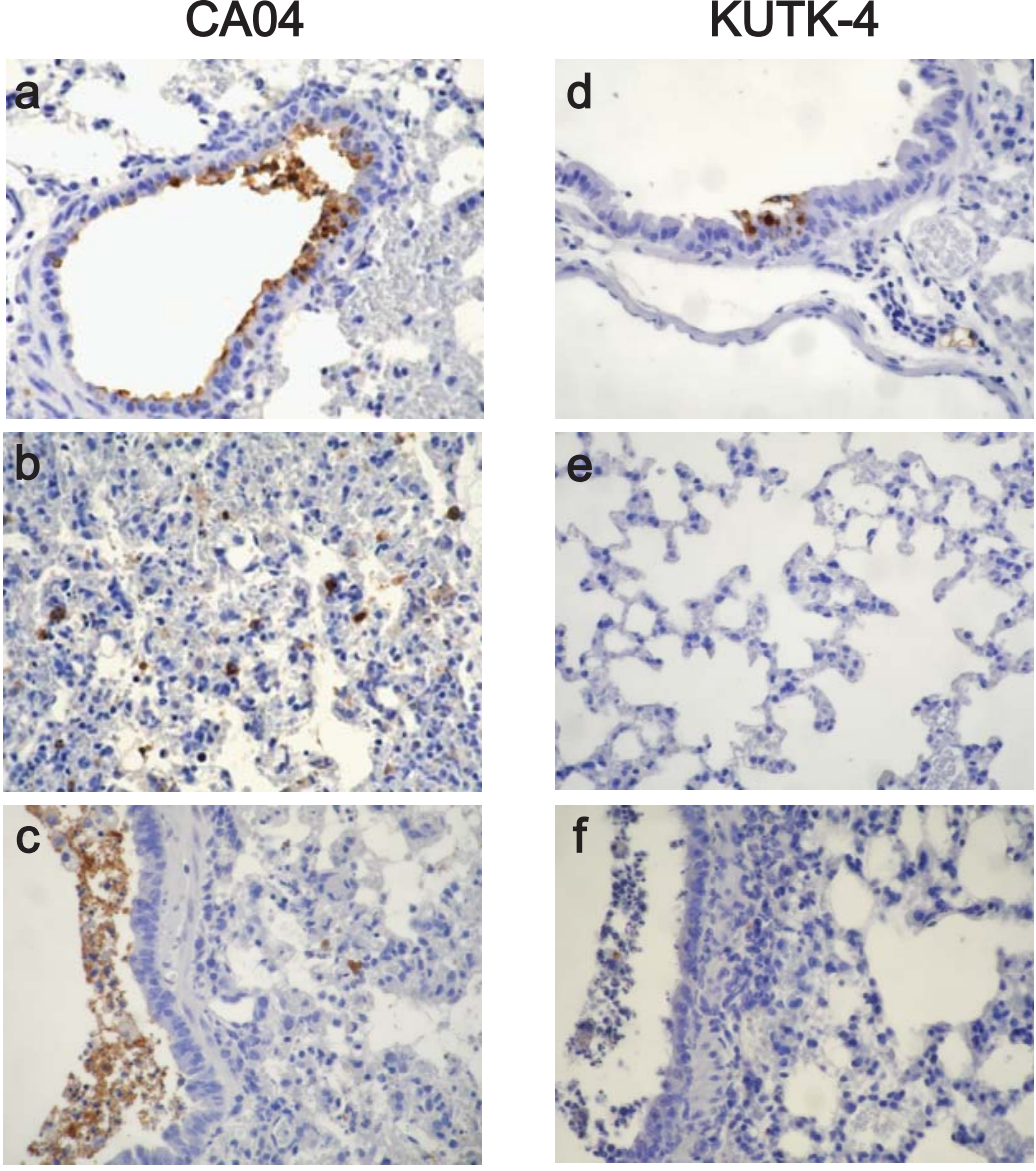


Uninfected

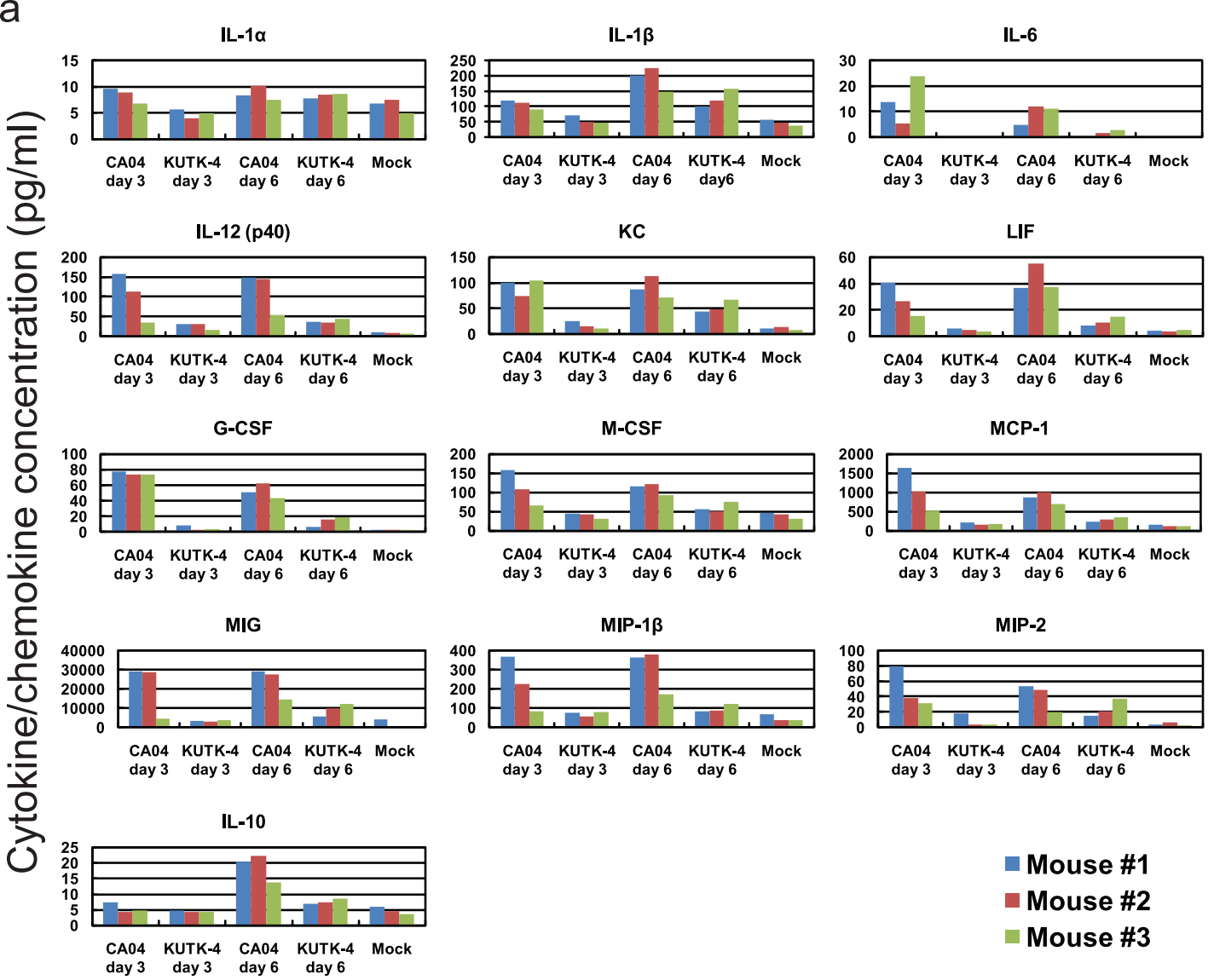




Supplementary Figure S3. Body weight changes in infected mice. Three mice per group were intranasally inoculated with 10^2, 10^3, 10^4, or 10^5 PFU (each in 50 µl) of CA04 (red) or KUTK-4 (blue), or undiluted virus ($10^{6.5}$ PFU for CA04 and $10^{6.6}$ PFU for KUTK-4). Body weights were monitored daily. Mice with body weight loss of more than 25% of pre-infection values were euthanized. The values are means ± SD from three mice.

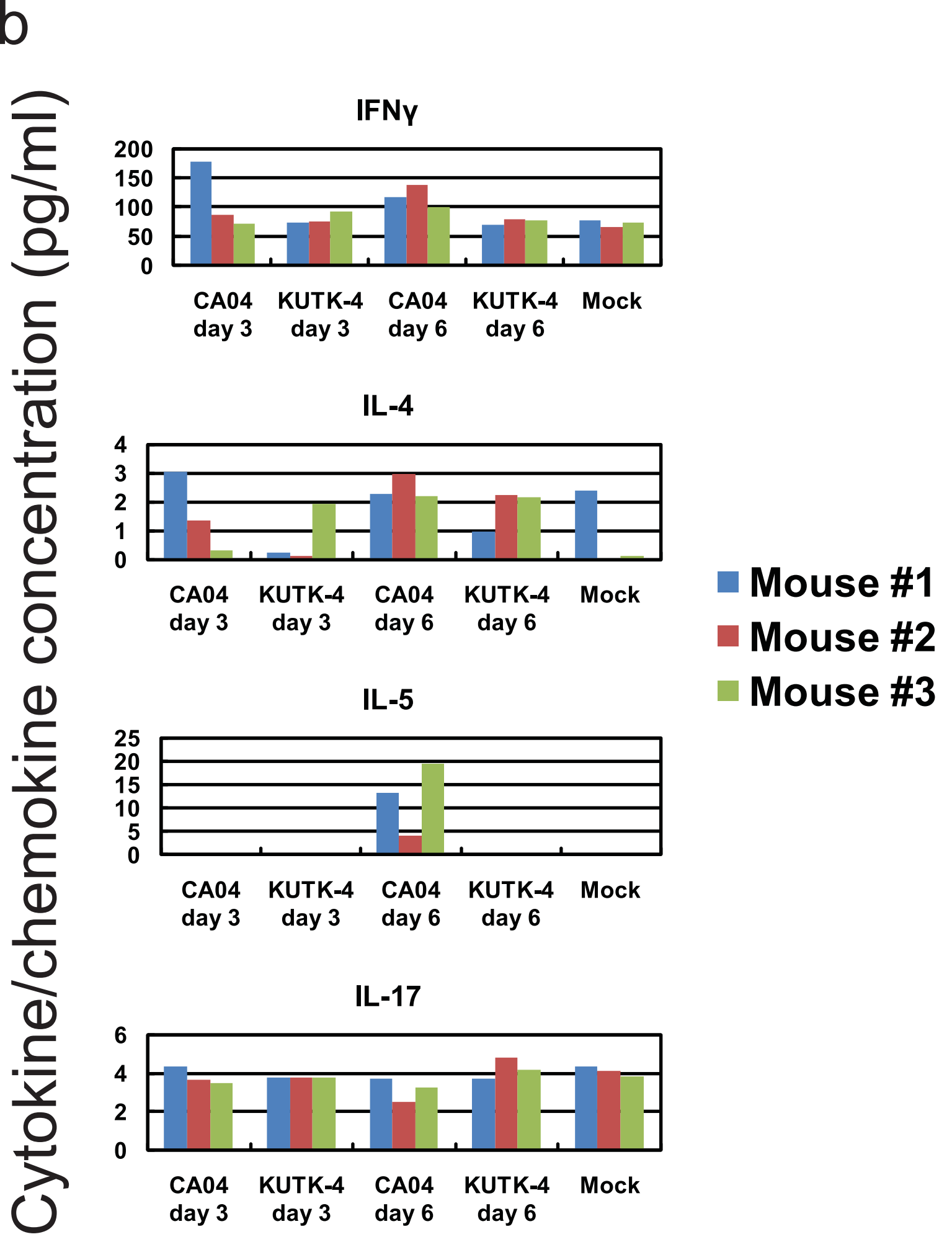


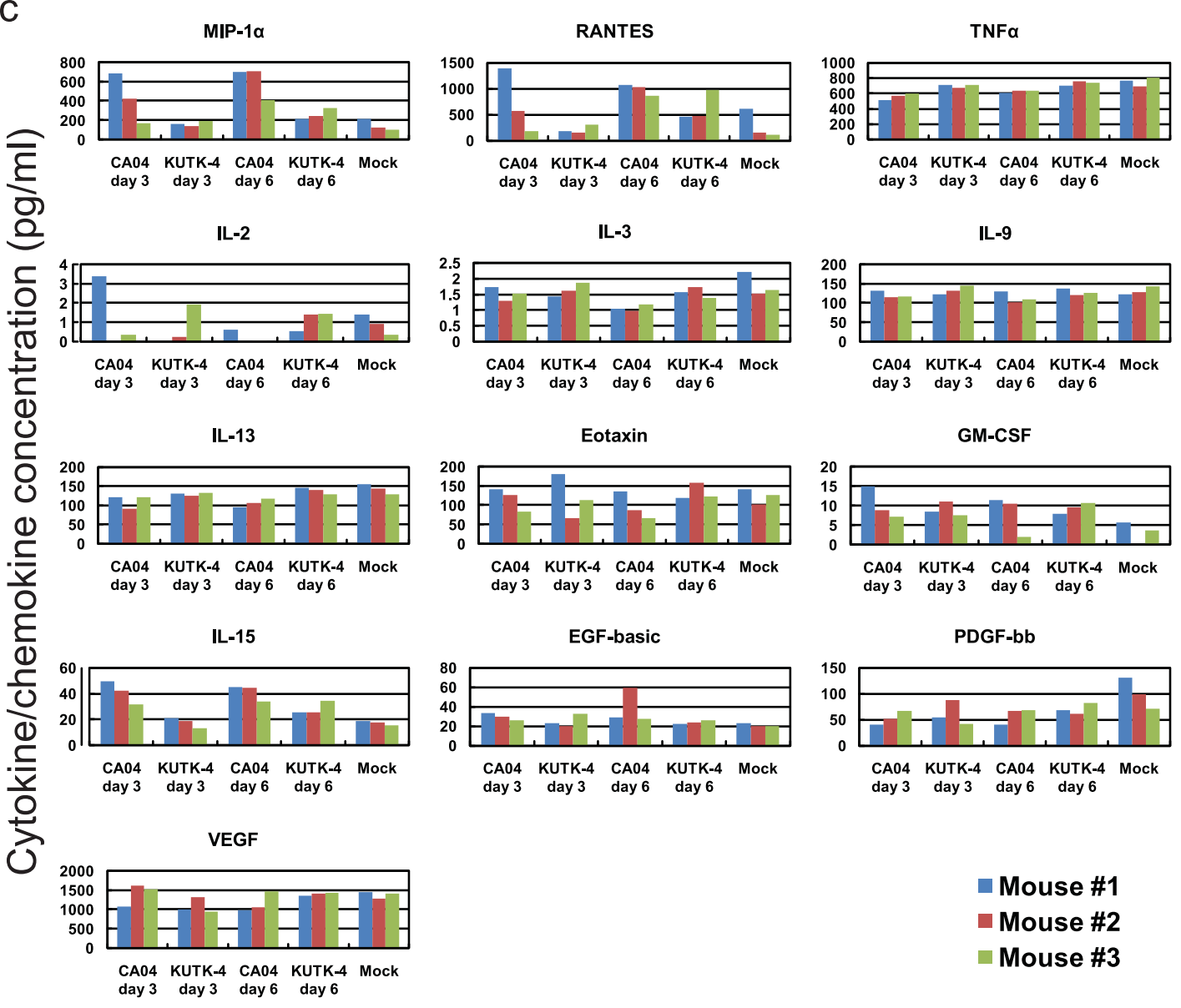
Supplementary Figure S4. Pathological findings in infected mice. Representative pathological findings for the lungs of mice infected with CA04 (**a-c**), or KUTK-4 (**d-f**). Infection with CA04 resulted in detectable viral antigen in bronchiolar epithelia and desquamated cells in the bronchiolar lumen on day 3 pi (**a**). Also, prominent alveolar thickening with scattered antigen-positive cells in the alveolus was observed (**b**). By day 6, epithelia were regenerative but accumulation of antigen-positive cell debris in the lumen was prominent (**c**). Upon infection with KUTK-4, a small number of viral antigen-positive cells was detected in the bronchial and bronchiolar epithelia on day 3 pi (**d**), but no viral antigen was detected in the alveolar area (**e**). On day 6 pi, accumulation of cell debris in the bronchiolar lumen with peribronchiolitis was observed, but viral antigens were rarely detected in these lesions (**f**).

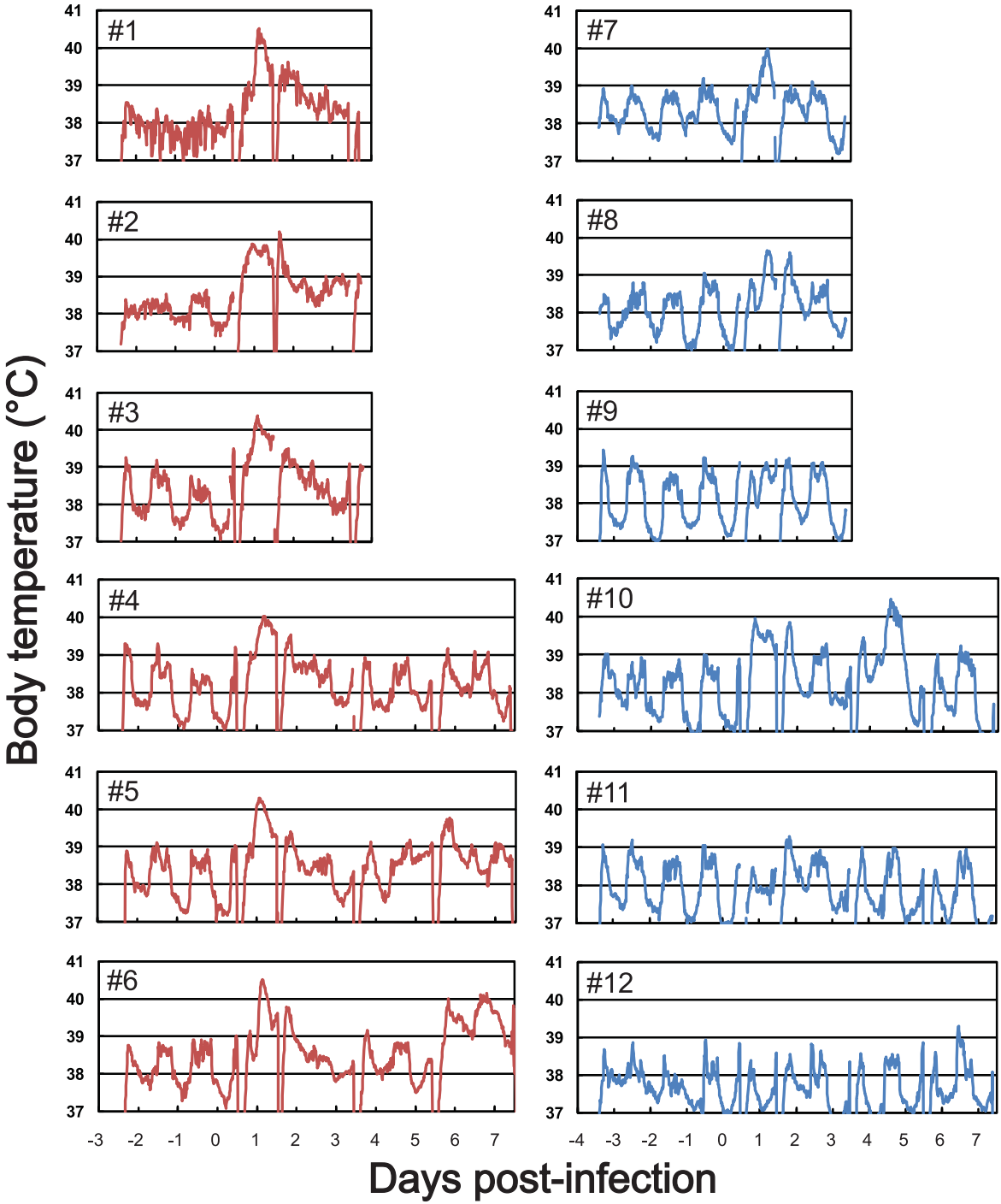


Supplementary Figure S5. Pro-inflammatory cytokine/chemokine responses in the lungs of infected mice. The concentrations of various cytokines/chemokines were measured in the lungs of mice by use of a protein array analysis with the Bio-Plex Mouse Cytokine 23-Plex and 9-Plex panels (Bio-Rad laboratories). IL-12 (p70) was not detected. IL-18 data are not available due to technical problem of the manufacturer.

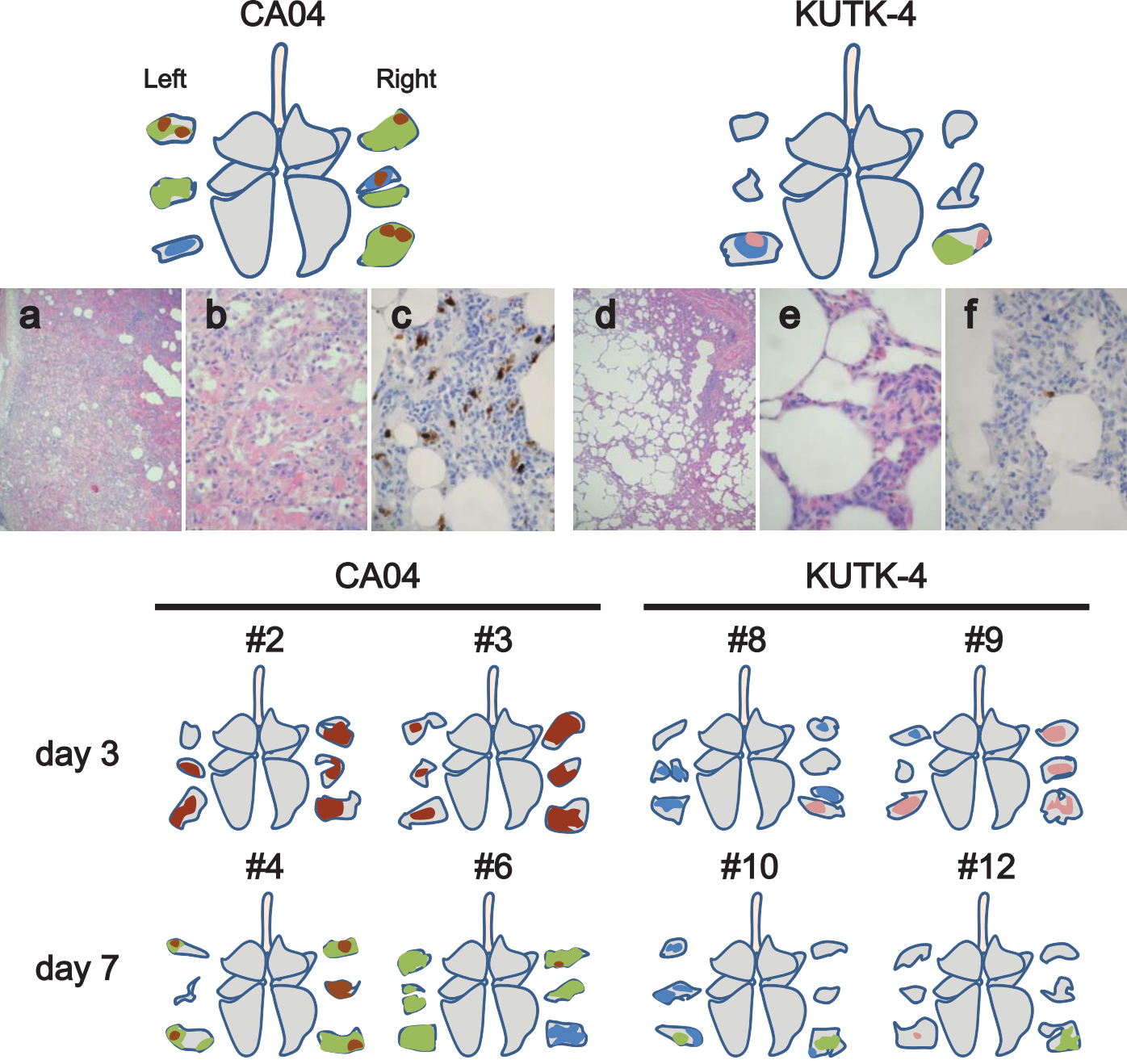
b


Cytokine/chemokine concentration (pg/ml)
IFNγ
200
150
100
50
0
CA04 day 3
KUTK-4 day 3
CA04 day 6
KUTK-4 day 6
Mock
IL-4
4
3
2
1
0
CA04 day 3
KUTK-4 day 3
CA04 day 6
KUTK-4 day 6
Mock
IL-5
25
20
15
10
5
0
CA04 day 3
KUTK-4 day 3
CA04 day 6
KUTK-4 day 6
Mock
IL-17
6
4
2
0
CA04 day 3
KUTK-4 day 3
CA04 day 6
KUTK-4 day 6
Mock
Mouse #1
Mouse #2
Mouse #3

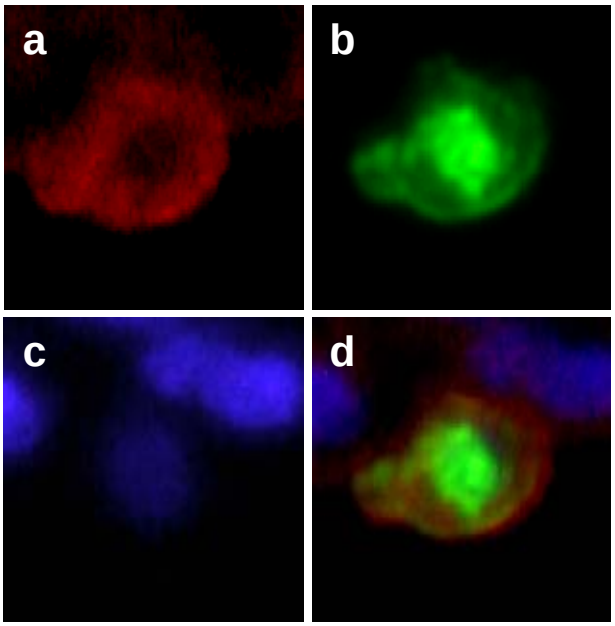

c
Cytokine/chemokine concentration (pg/ml)
MIP-1α
RANTES
TNFα
IL-2
IL-3
IL-9
IL-13
Eotaxin
GM-CSF
IL-15
EGF-basic
PDGF-bb
VEGF
CA04 day 3
KUTK-4 day 3
CA04 day 6
KUTK-4 day 6
Mock
Mouse #1
Mouse #2
Mouse #3



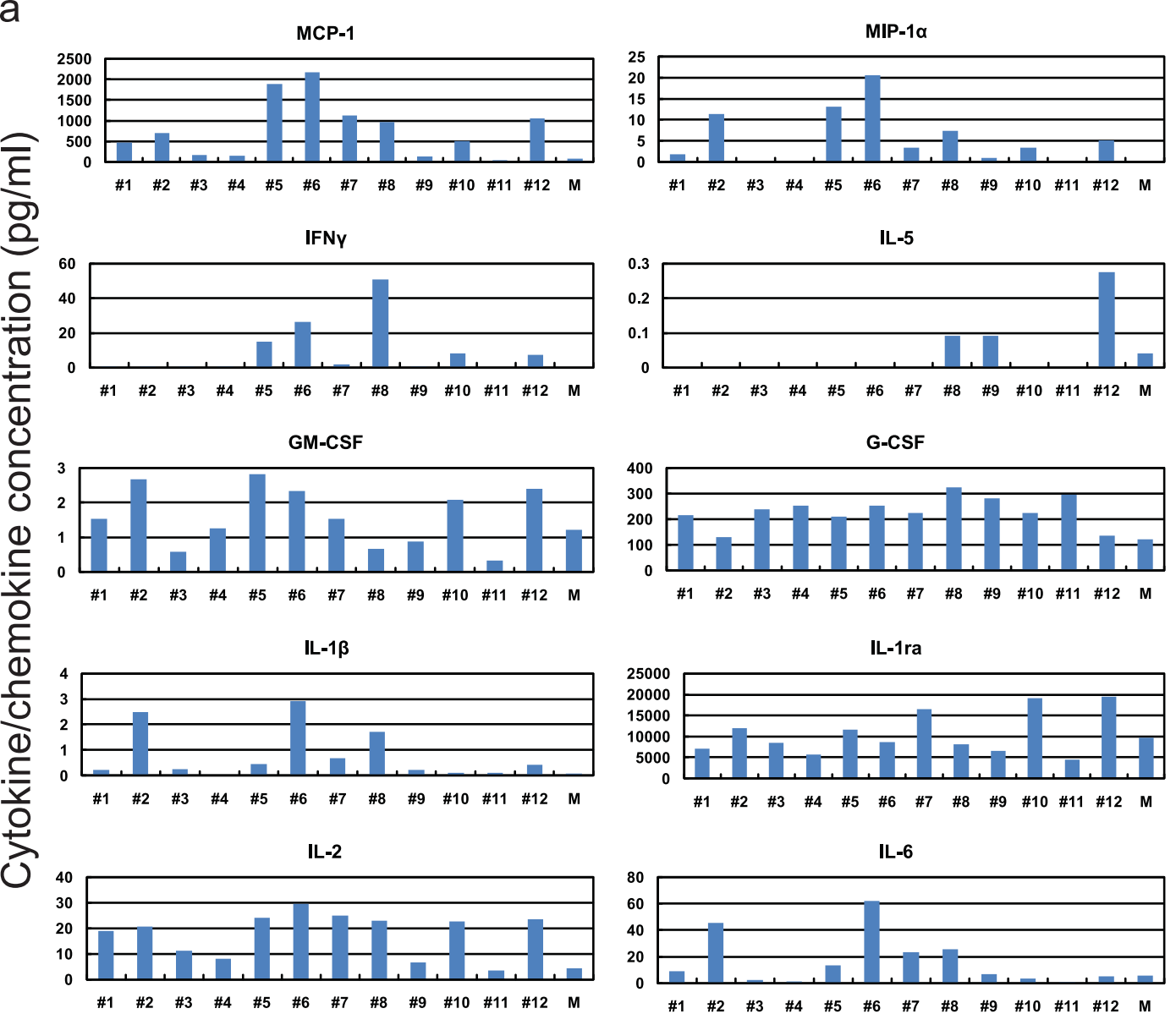
Supplementary Figure S6. Body temperatures of infected cynomolgus macaques. Six macaques per group were inoculated with $10^{7.4}$ PFU (total volume: 6.7 ml) of CA04 (red, #1-6) or KUTK-4 (blue, #7-12) through multiple routes (see Supplementary materials and methods). Temperatures were monitored every 15 minutes by telemetry probes implanted in the peritoneal cavities. The periodic sharp reduction in body temperatures on days 0, 1, 3, and 7 was caused by anesthesia required for sampling. Monkeys #1-3 and #7-9 were euthanized on day 3.



Supplementary Figure S7. Pathological findings in infected cynomolgus macaques. Shown are representative pathological findings in the lungs of cynomolgus macaques on day 7 post infection with CA04 (macaque #5, **a-c**) or KUTK-4 (macaque #11, **d-f**) (upper portion). Schematic figures summarize the distribution of lesions, with or without viral antigen, in the lungs of the remaining virus-inoculated macaques. Colors: green, severe lung lesions where alveolar spaces were filled with edema fluid, inflammatory cells, or cell debris; brown, severe lung lesions containing moderate to many viral antigen-positive cells; pink, mild lung lesions containing a few viral antigen-positive cells; blue, lung lesions where severe alveolar wall thickening was prominent, but air spaces were preserved. (**a**) Alveolar spaces were not clear because of inflammatory exudate. (**b**) Large areas of affected lung contained accumulated cell debris, inflammatory infiltrates, fibrin, and edema fluid; alveolar walls were thickened by infiltration of inflammatory cells. (**c**) Viral antigen-positive cells were detected extensively in lung lesions in some areas. (**d**) In most areas, alveolar spaces were still clear, although thickening of the alveolar walls was apparent. (**e**) Most lung lesions consisted of thickening of alveolar walls by mononuclear cells. (**f**) A few antigen-positive cells were detected in lung lesions.

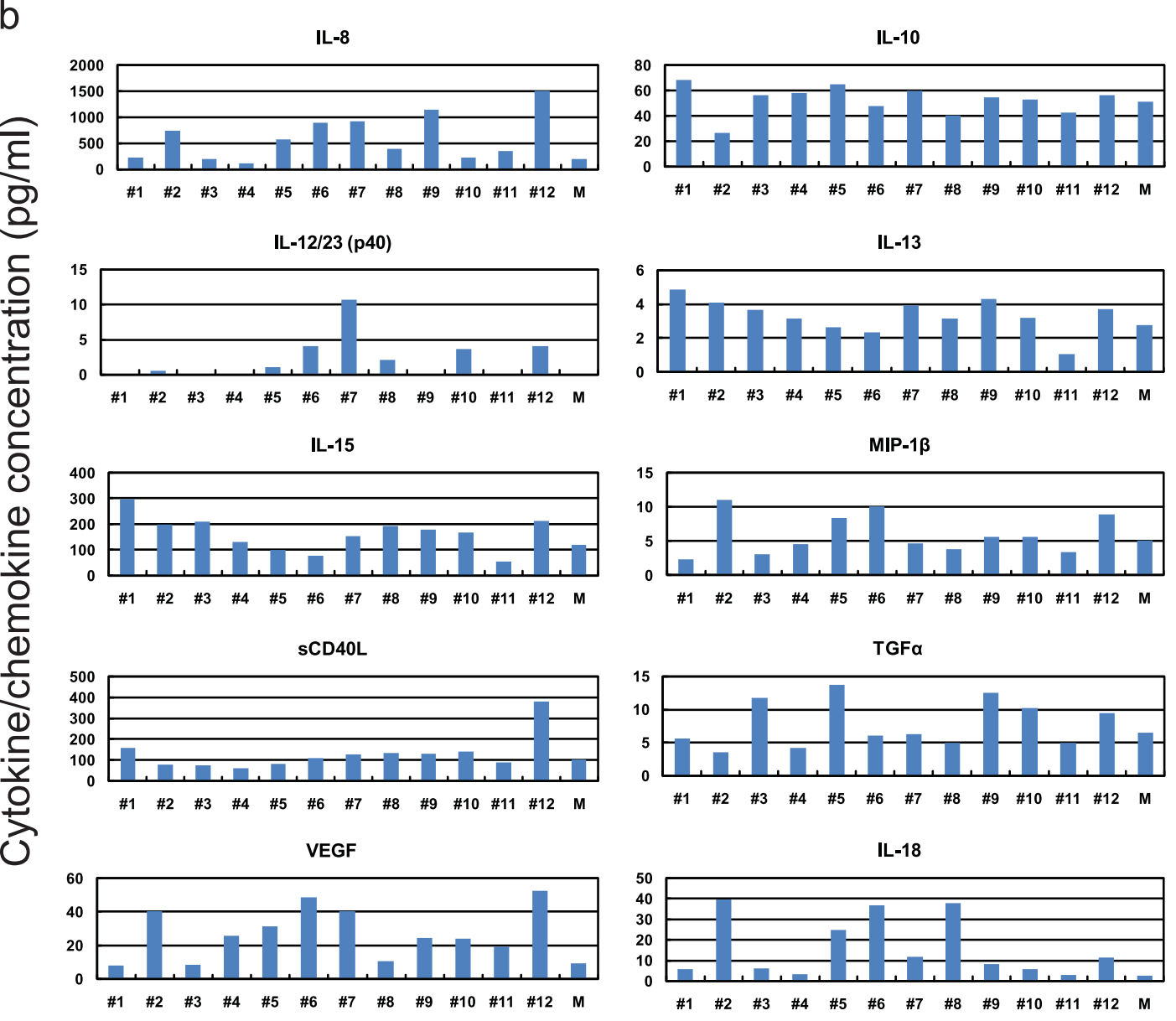


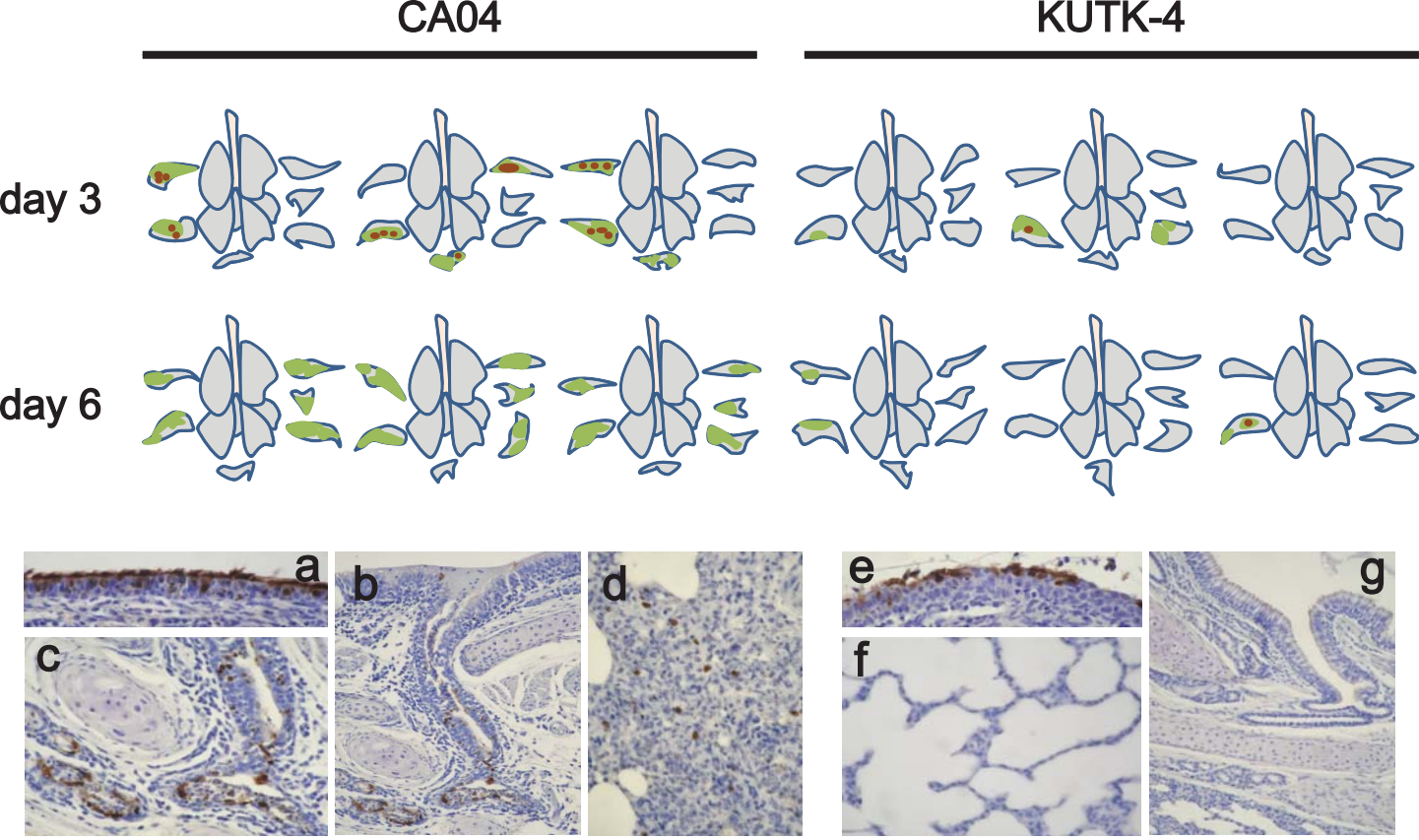
Supplementary Figure S8. Detection of viral antigens in type II pneumocytes in the lungs of CA04-infected cynomolgus macaques. On day 3 post-infection, cells were stained with anti-cytokeratin (N1590, DAKO) antibody (**a**; red) and anti-influenza (H1N1) antibody (**b**; green). The nucleus was stained with DAPI (**c**). Considerable amounts of viral antigen were detected in type II pneumocytes (**d**).



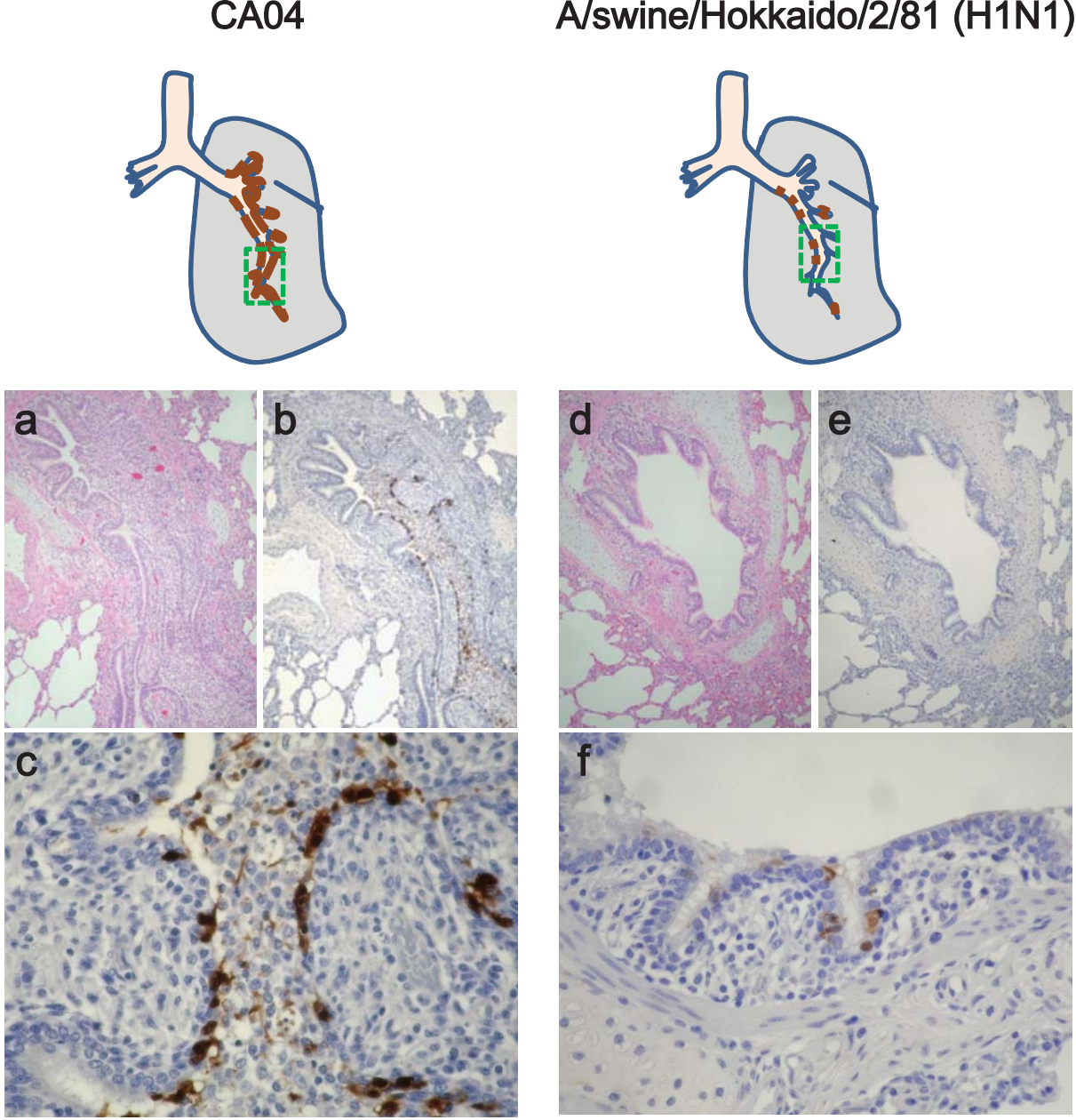
Supplementary Figure S9. Pro-inflammatory cytokine/chemokine responses in the lungs of infected cynomolgus macaques. The concentrations of various cytokines/chemokines in the lungs of infected cynomolgus macaques on day 3 post-infection were measured by protein array analysis with the MILLIPLEX MAP Non-human Primate Cytokine/Chemokine Panel - Premixed 23-Plex (Millipore, Bedford, MA). We did not detect IL-4, IL-17, or TNFα in the lungs or G-CSF and IL-4 in the sera.

b


Cytokine/chemokine concentration (pg/ml)
IL-8
2000
1500
1000
500
0
#1 #2 #3 #4 #5 #6 #7 #8 #9 #10 #11 #12 M
IL-10
80
60
40
20
0
#1 #2 #3 #4 #5 #6 #7 #8 #9 #10 #11 #12 M
IL-12/23 (p40)
15
10
5
0
#1 #2 #3 #4 #5 #6 #7 #8 #9 #10 #11 #12 M
IL-13
6
4
2
0
#1 #2 #3 #4 #5 #6 #7 #8 #9 #10 #11 #12 M
IL-15
400
300
200
100
0
#1 #2 #3 #4 #5 #6 #7 #8 #9 #10 #11 #12 M
MIP-1β
15
10
5
0
#1 #2 #3 #4 #5 #6 #7 #8 #9 #10 #11 #12 M
sCD40L
500
400
300
200
100
0
#1 #2 #3 #4 #5 #6 #7 #8 #9 #10 #11 #12 M
TGFα
15
10
5
0
#1 #2 #3 #4 #5 #6 #7 #8 #9 #10 #11 #12 M
VEGF
60
40
20
0
#1 #2 #3 #4 #5 #6 #7 #8 #9 #10 #11 #12 M
IL-18
50
40
30
20
10
0
#1 #2 #3 #4 #5 #6 #7 #8 #9 #10 #11 #12 M



Supplementary Figure S10. Pathological findings in infected ferrets. CA04-infected ferret lungs showed severe and wide lung lesions with viral antigen on day 3 pi and without viral antigen on 6 day pi. KUTK-4-infected ferret lungs showed limited lung lesions with viral antigen on days 3 and 6 pi. Representative pathological findings of nasal mucosa and lungs of CA04- (**a**-**d**), and KUTK-4- (**e**-**g**) infected ferrets on days 3 and 6 pi. (**a**) Extensive viral antigen present at the nasal epithelium on day 3 pi in CA04-infected ferret. (**b**) and (**c**) In the lungs, viral antigen was mainly detected in the peribroncial glands with severe peribronchitis and bronchopneumonia (**d**) Sparse viral antigen was detected within alveolar lesions. (**e**) Extensive viral antigen expression at the nasal mucosa on day 3 pi in KUTK-4-infected ferret. (**f**) Most of the lung was not affected by viral infection in KUTK-4-infected ferrets. (**g**) Most of the peribronchial gland appeared normal in KUTK-4-infected ferret lungs at day 6 pi. Colors: green, severe lung lesions where alveolar spaces were filled with edema fluid, inflammatory cells, or cell debris; brown, severe lung lesions containing moderate to many viral antigen-positive cells.



Supplementary Figure S11. Pathological findings in infected miniature pigs. Shown are representative pathological findings for the lungs of miniature pigs on day 3 post infection with CA04 (miniature pig #1, **a-c**) or A/swine/Hokkaido/2/81 (H1N1) (miniature pig #5, **d-f**). The distribution of viral antigen (brown) is shown in the schematic figures. (**a**) The lumens of the bronchus and bronchioles were filled with inflammatory infiltrates. (**b**) Viral antigen was detected along bronchus and bronchiole. (**c**) Viral antigen was mainly detected in epithelial cells and desquamated cells. (**d**) The bronchial lumen remained clear with limited inflammatory reactions. (**e**) and (**f**) Few antigen-positive cells were detected at the epithelium, with minimum inflammatory reaction. The areas delineated by green boxes in the schematic diagrams correspond to the histopathological sections.

Supplementary Table 1. Virus titres in organs of infected mice[a].

Virus		Virus titres (mean log_{10} PFU ± SD/g) in:	
		Nasal turbinates	Lungs
A/California/04/09 (H1N1)	Day 3	6.6±0.2	7.8±0.03
	Day 6	5.4±0.6	6.8±0.01
A/Netherlands/603/09 (H1N1)	Day 3	6.6±0.2	6.8±0.3
	Day 6	5.5±0.2	6.2±0.2
A/Wisconsin/WSLH049/09 (H1N1)	Day 3	6.7±0.2	7.2±0.2
	Day 6	6.3±0.2	6.5±0.1
A/Wisconsin/WSLH34939/09 (H1N1)	Day 3	7.1±0.2	7.7±0.2
	Day 6	5.9±0.3	6.9±0.5
A/Osaka/164/09 (H1N1)	Day 3	6.3±0.7	7.2±0.1
	Day 6	3.8±1.3	6.5±0.4
A/Kawasaki/UTK-4/09 (H1N1)	Day 3	6.3±0.2	6.4±0.3
	Day 6	5.0±0.3	4.6±0.4

[a]BALB/c mice were intranasally infected with 10^5 PFU (50 µl) of virus. Three mice from each group were euthanized on days 3 and 6 pi for virus titration. None of the viruses tested was recovered from the spleens, kidneys, brains, colons, or livers of infected animals.

Supplementary Table 2. Virus titres in respiratory swabs from infected cynomolgus macaques[a].

		Virus titre (log_{10} PFU/ml) of animals infected with:											
		A/California/04/09 (H1N1)						A/Kawasaki/UTK-4/09 (H1N1)					
animal ID		#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	#11	#12
Nasal swab	Day 1	5.8	1.0	1.5	4.7	4.5	2.6	3.0	2.9	1.6	3.6	2.4	3.6
	Day 3	5.2	2.1	2.5	3.7	2.6	3.3	2.8	3.2	2.4	4.1	1.5	3.3
	Day 5				4.7	4.6	3.4				1.3	3.6	2.5
	Day 7				-[b]	3.5	-				-	-	5.0
Tracheal swab	Day 1	3.4	2.3	3.6	2.3	3.5	2.0	1.3	1.3	-	2.0	2.3	2.1
	Day 3	4.3	-	2.6	2.6	2.4	2.0	1.0	1.8	-	4.0	-	-
	Day 5				3.5	2.5	3.7				5.6	-	-
	Day 7				-	2.0	-				3.4	-	2.6
Bronchial brush	Day 1	2.9	2.4	3.7	2.2	3.3	-	1.5	-	-	-	-	-
	Day 3	3.5	-	-	3.1	-	-	-	1.5	-	-	-	-
	Day 5				4.4	2.4	1.8				4.4	-	1.3
	Day 7				-	-	-				1.5	-	4.4

[a]Cynomolgus macaques were inoculated with $10^{7.4}$ PFU of virus (6.7 ml) through multiple routes. Nasal and tracheal swabs and bronchial brush samples were collected every other day for virus titration.

[b]-, virus not detected (detection limit: 1.0 log_{10} PFU/ml).

Diagonal lines: not applicable, animals euthanized on day 3 pi.

Supplementary Table 3. Virus titres in respiratory organs of infected ferrets[a].

Virus		Virus titres (mean log_{10} PFU ± SD/g) in:		
		Nasal turbinates	Trachea	Lungs
A/California/04/09 (H1N1)	Day 3	6.7±0.7	5.9±0.4	3.53, 4.12
	Day 6	2.40	3.1±0.1	2.95
A/Netherlands/603/09 (H1N1)	Day 3	7.3±0.6	6.0±1.7	5.15
	Day 6	3.26, 4.77	4.36, 5.14	-[b]
A/Wisconsin/WSLH049/09 (H1N1)	Day 3	7.8±0.6	6.0±0.9	6.49, 3.23
	Day 6	4.3±1.1	4.57, 3.40	5.48
A/Wisconsin/WSLH34939/09 (H1N1)	Day 3	8.3±0.1	4.6±0.3	4.5±1.8
	Day 6	4.5±1.0	3.8±1.4	3.6±1.1
A/Osaka/164/09 (H1N1)	Day 3	6.9±1.0	6.4±1.0	6.8±0.8
	Day 6	-	-	-
A/Kawasaki/UTK-4/09 (H1N1)	Day 3	6.5±0.5	2.45, 3.81	-
	Day 6	-	3.18	-

[a]Ferrets were intranasally infected with 10^6 PFU (500 µl) of virus. Three ferrets from each group were euthanized on days 3 and 6 pi for virus titration. When virus was recovered from all three animals, average titres are presented. When virus was not recovered from all three ferrets, individual titres were recorded. None of the viruses tested was recovered from the spleens, kidneys, brains, intestines, or livers of infected animals.

[b]-, virus not detected (detection limit: 2.3 log_{10} PFU/g).

Supplementary Table 4. Virus titres in nasal swabs of inoculated and contact ferrets[a]

Virus			Virus titres (log_{10} PFU/ml) in nasal swabs				
			Day 1	Day 3	Day 5	Day 7	Day 9
A/California/04/09 (H1N1)	Pair 1	*i*	7.1	4.0	-[b]	-	-
		c	-	6.8	4.3	-	-
	Pair 2	*i*	7.1	5.3	3.4	-	-
		c	-	6.2	4.0	-	-
	Pair 3	*i*	5.9	5.3	3.9	-	-
		c	-	6.4	5.9	2.1	-
A/Kawasaki/UTK-4/09 (H1N1)	Pair 4	*i*	6.6	5.3	4.3	-	-
		c	-	5.0	4.5	2.5	-
	Pair 5	*i*	6.1	5.9	1.3	-	-
		c	-	-	-	-	-
A/Victoria/03/75 (H3N2)	Pair 6	*i*	6.3	3.9	2.0	-	-
		c	-	-	6.0	3.7	2.5
	Pair 7	*i*	5.8	2.3	-	-	-
		c	-	-	-	-	2.3
A/duck/Alberta/35/76 (H1N1)	Pair 8	*i*	-	2.8	2.6	-	-
		c	-	-	-	-	-
	Pair 9	*i*	-	2.3	-	-	-
		c	-	-	-	-	-
	Pair 10	*i*	-	4.9	3.7	4.0	-
		c	-	-	-	-	-

[a]For pairs of ferrets, one animal was intranasally inoculated with 10^6 PFU of virus (500 µl) (inoculated ferret, *i*) and one day later, a naïve ferret was placed in an adjacent cage (contact ferret, *c*). Nasal swabs were collected from inoculated and contact ferrets every other day for virus titration.

[b]-, virus not detected (detection limit: 1.3 log_{10} PFU/ml).

Supplementary Table 5. Virus titres in organs of infected miniature pigs[a].

	Virus titres (log_{10} PFU/g) of animals infected with:			
	A/California/04/09 (H1N1)		A/swine/Hokkaido/2/81 (H1N1)	
Animal ID	#1	#2	#5	#6
Nasal mucosa	6.7	5.0	5.1	4.8
Oro/nasopharynx	3.1	3.3	6.8	5.0
Tonsil	3.2	-[b]	4.5	4.4
Trachea	6.3	5.5	5.8	5.3
Bronchus (right)	5.6	6.1	5.9	6.5
Bronchus (left)	6.7	6.5	5.4	6.3
Lung (upper right)	7.8	6.6	6.1	4.5
Lung (middle right)	7.5	6.7	6.1	5.5
Lung (lower right)	6.4	6.8	5.3	4.5
Lung (upper left)	6.8	6.4	6.8	5.1
Lung (middle left)	8.0	7.6	4.7	5.5
Lung (lower left)	6.2	7.4	5.5	4.7
Ileum	-	-	3.5	-
Jejunum	-	-	2.8	-

[a]Specific-pathogen free miniature pigs were intranasally infected with $10^{6.2}$ PFU (1 ml) of virus. Two animals from each group were euthanized on day 3 pi for virus titration. No virus was recovered from heart, spleen, kidneys, liver, duodenum, rectum, bladder, cerebrum, cerebellum, or brain stem.

[b]-, virus not detected (detection limit: 2.0 log_{10} PFU/g).

Supplementary Table 6. Virus titres in nasal swabs from infected miniature pigs[a].

		Virus titers (log_{10} PFU/ml) of animals infected with:							
		A/California/04/09 (H1N1)				A/swine/Hokkaido/2/81 (H1N1)			
animal ID		#1	#2	#3	#4	#5	#6	#7	#8
Nasal swab	day 1	5.6	6.5	6.4	6.1	6.3	5.3	3.6	4.1
	day 2	6.5	6.5	7.4	6.7	5.5	5.5	5.1	5.6
	day 3	5.7	5.3	7.2	5.3	5.0	4.4	4.7	5.5
	day 4			3.7	3.6			4.9	4.6
	day 5			4.5	5.4			2.7	3.2
	day 6			4.3	5.3			2.8	3.2
	day 7			3.3	3.4			1.3	1.6
	day 8			-[c]	-			-	-
	day 9			-	-			-	-

[a]Miniature pigs were intranasally infected with $10^{6.2}$ PFU (1 ml) of virus. Nasal swabs were collected daily for virus titration.

[b]-, virus not detected (detection limit: 1.0 log_{10} PFU/ml).

Diagonal lines: not applicable, animals euthanized on day 3 pi.

Supplementary Table 7. Virus susceptibility to antiviral compounds in cell culture.

	IC_{90}		
	A/California/04/09 (H1N1)	A/Kawasaki/UTK-4/09 (H1N1)	A/Kawasaki/UTK-23/08 (H1N1)
Oseltamivir carboxylate[a]	10.56[c]	2971.30	5.58
Zanamivir	17.67	42.33	21.93
R-125489[b]	4.24	11.70	10.17
T-705	0.16	0.23	0.13

[a]Oseltamivir carboxylate is the active form of oseltamivir.

[b]R-125489 is the active form of CS-8958.

[c]IC_{90} value: mean μg/ml or nM of triplicate reactions for T-705 and the other compounds tested, respectively.

Supplementary Table 8. Virus sensitivity in neuraminidase inhibition assays.

	IC_{50}			
	A/California/04/09 (H1N1)	A/Osaka/164/09 (H1N1)	A/Kawasaki/UTK-4/09 (H1N1)	A/Kawasaki/UTK-23/08 (H1N1)
Oseltamivir carboxylate[a]	0.96[c]	1.6	1313	1.88
Zanamivir	0.32	0.43	0.79	0.36
R-125489[b]	0.41	0.44	0.34	0.20

[a]Oseltamivir carboxylate is the active form of oseltamivir.

[b]R-125489 is the active form of CS-8958.

[c]IC_{50} value: mean nM of duplicate reactions.