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ORIGINAL RESEARCH

Keratins provide virus-dependent protection or predisposition to injury in coxsackievirus-induced pancreatitis

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Abstract: Keratins 8 and 18 (K8/K18) are the two major intermediate filament proteins in hepatocytes and pancreatic acinar cells. Acinar cell keratins are organized as cytoplasmic and apicolateral filaments. An important role of hepatocyte K8/K18 is to maintain cellular integrity, while this cytoprotective function of K8/K18 is not evident in the pancreas since keratin-deficient mice cope well with pancreatitis models. To further study the roles of keratins in the exocrine pancreas, we used coxsackievirus B4-models, CVB4-V and CVB4-P, to induce severe acute/chronic pancreatitis and acute pancreatitis, respectively, in K8-null (which lack acinar keratins) and K18-null (which lack cytoplasmic keratins) mice. Despite similar virus titers in all mice, CVB4-V resulted in 40% mortality of the K8-null mice 14 days post-infection compared to no lethality of WT and K18-null mice. In contrast, K8-null mice were far less susceptible to CVB4-P-induced damage as determined by histology and serology analysis, and they recover faster than WT and K18-null mice. After CVB4 virus infection, keratins aggregated during acinar degranulation, and K8/K18 site-specific phosphorylation was observed during degranulation and recovery. Hence, keratins significantly affect CVB4 virulence, positively or negatively, depending on the virus subtype and keratin makeup, in a virus replication-independent manner.

Keywords: keratin, pancreatitis, coxsackievirus

Introduction

Keratin 8 (K8) and keratin 18 (K18) are the two major intermediate filament proteins expressed in hepatocytes and acinar cells of the exocrine pancreas. Pancreatic acinar K8/K18 can be topographically divided into apicolateral keratins (AL- keratins), located beneath the luminal space, and cytoplasmic filaments (C-keratins) that are spread across the cytoplasm.[1,2] Mouse but not human acinar cells contain K19, in addition to K8/K18, and smaller amounts of K20 which are expressed only as AL-keratins under baseline conditions but can form C-keratins during recovery from pancreatitis.[1,2] K23 is a novel keratin that is induced by histone deacetylase inhibitors during differentiation in pancreatic cancer cells[3] and can be considered a member of the simple epithelial subgroup of keratins.[4]

The functions of pancreatic K8/K18 are poorly understood and the cytoprotective function of K8/K18 in the liver is not evident in the pancreas. For example, transgenic mice that lack K8 (no acinar cell keratins) or K18 (only AL-keratins), or that overexpress human K18 R90C (intact AL-keratins but fragmented C-keratins) show dramatic predisposition to liver injury.[5–7] In contrast, K8/K18 are dispensable in the pancreas when tested using two established mouse pancreatitis models, supramaximal caerulein injections and a choline-deficient ethionine- supplemented

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diet (CDD).[8] Pancreatic acini and acinar cells can also be isolated with good viability from these mice in contrast to liver hepatocytes that are extremely fragile upon isolation by liver perfusion.[1,6,8] Microarray analysis of K8-null and WT pancreata show, however, that multiple genes are differentially regulated, such as the regenerating islet-derived protein II (Reg-II) which is dramatically upregulated in the pancreas of K8-null mice and in transgenic mice harboring human keratin mutations.[9] In addition, mouse pancreata cope well with modest levels of K8/K18 overexpression, while high expression is toxic to the pancreas and leads to atrophy and increased keratin phosphorylation at older age, while keratin overexpression in the liver is tolerated well.[10–11] Together these studies suggest that keratins in the liver and pancreas have different functions.

In order to further characterize potential cytoprotective functions of keratins in the pancreas, we used two strains of the picornavirus coxsackievirus B4 (CVB4-V and CVB4-P) to induce pancreatitis[12,13] in keratin-related mouse models. CVB4-P induces a mild acute pancreatitis with subsequent repair, while CVB4-V results in fat replacement, fibrosis of the pancreas and is a model of severe acute pancreatitis with lethal outcome.[13] The subset of mice that survive CVB4-V infection develop chronic pancreatitis.[13] Both viruses replicate in the pancreas (the CVB4-V virus also replicates in the heart) and cause hyperamylasemia, with virus levels peaking 3 days after infection.[14] In the study reported herein, CVB4 infection of K8-null and K18-null mice showed that K8-null mice are more sensitive to lethality by CVB4-V but much less susceptible to CVB4-P-induced acute pancreatitis from which the K8-null mice recover much faster than WT and K18 mice.

Materials and methods

Antibodies

Primary antibodies that were used include: horse (ho) anti-CVB4 antibody VR-1035 (ATTC); rat Troma I [anti-mouse (m)K8] and rat Troma III (anti-mK19) (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA];[15] rabbit 8592 (anti-h,mK8/K18);[16] mouse 5B3 [phospho-(p)S438]; mouse LJ4 (anti-mK8 pS80)[17] and rabbit 8250 (anti-mK18 pS35).[18] Nuclei were stained using Toto-1 (Molecular Probes).

Mice

The K8-null (FVB/n background) and K18-null mice (Balb/C and C57BL/6 mixed background) were genotyped as described.[19,20] Heterozygous (+/–) mice were inter-bred to derive null (–/–), heterozygous and wild type (K8WT or K18WT depending on the starting +/– strain used) mice. Animals were maintained and virus infections performed in accordance with institutional regulations.

Virus infection

CVB4-V and CVB4-P strains were propagated and kept in culture.[14] Injection (i.p.) of coxsackievirus to age and sex-matched mice was done with a 26G needle using 1×10^4 pfu/mouse in 200 to 300 μL of sterile PBS (without calcium, pH 7.4). All mice used for CVB4-P experiments were 2 to 3 months old male (except for 1 female of 4 mice for the K18 strain at the CVB4-P14 time point), while all mice used for the CVB4-V experiments were 4 months old and sex matched as availability allowed (Supplemental Table 1). Control mice were injected with PBS alone. All mouse cages underwent daily changes of H_2O containing 10% glucose and 1 mg/mL pancreatin (Sigma). For CVB4-V infection, mice were monitored daily for survival, or sacrificed by CO_2 inhalation on day 2 (V2) or on day 14 (V14) post infection (sooner if moribund). Mice infected with CVB4-P virus were sacrificed 2 (P2), 6 (P6) or 14 (P14) days post-infection. Upon sacrifice, blood was drawn by cardiac puncture followed by serum analysis of amylase and lipase. Pancreata were excised and a) fixed in 10% buffered formalin for hematoxylin and eosin (H&E) staining, b) fresh-frozen in optimum cutting temperature compound (O.C.T.; Miles, Elkhart, IN) or frozen in liquid N_2 for subsequent virus-titer studies and biochemical analysis. Liver and heart samples were also obtained for H&E to study damage in these organs.

Serum analysis, histology, tissue grading and microscopy

Serum amylase and lipase were assayed using an Express Plus instrument (Bayer; Tarrytown, NY). Formalin-fixed tissues were processed and stained by H&E (Histotec Laboratories; Hayward, CA). Pancreatitis severity was assessed using a relative histologic score that reflected acinar degranulation, levels of fat or inflammation (Table 3A), on examination of H&E-stained sections in a blinded fashion. For immunofluorescence studies, OCT-embedded pancreata were sectioned (6 μm) then fixed using cold acetone (–20 °C) and stained as described.[17] Samples were viewed and images taken with an MRC 1024 laser confocal scanning microscope (Bio-Rad; Hercules, CA) connected to a Nikon Eclipse TE300 inverted microscope with analysis using a Lasersharp 2.0 program. Image panels were compiled using Adobe Photoshop and Adobe Illustrator.

Statistical analysis

Numerical data were compared using *t*-test or Wilcoxon-Kruskal-Wallis test and the JMP 3.1 program (DataViz, Trumbull, CT) or Microsoft Excel. Data are presented as means and standard error of the mean (SEM) or standard deviation (SD). For the lethality experiments, results were analyzed using Fisher's exact test.

Results

Genotype-independent virus replication in mouse strains, and increased mortality in K8-null mice after virus infection

To assure that CVB4-P and CVB4-V were able to replicate similarly in the K8-null and K18-null mouse strains, pancreatic virus titers were analyzed on days 2, 6 and 14 after CVB4-P infection and days 2 and 14 after CVB4-V infection. The CVB4-P and CVB4-V viruses replicated similarly in all mice at levels similar to other mouse strains[13] (eg, the virus peaked at day 2 and declined on days 6 and 14; Figure 1). CVB4-P virus titers on day 14 were nearly zero in all mice (not shown). Titers for the heterozygous genotype mice were either not measured or close to those of WT and null mice (not shown). The presence or lack of keratin filaments does not affect coxsackievirus replication in the pancreas.

To study a model of severe acute/chronic pancreatitis, mice were injected i.p. with CVB4-V virus and monitored for 14 days. CVB4-V has been shown to lead to significant morbidity and mortality[13] which was observed preferentially in the K8-null mice (40% of the mice died or needed to be sacrificed by day 14, $p < 0.001$ when comparing K8-null to K8 WT and K8 +/– mice; Table 1). In contrast to K8-null mice, all K18-null mice, K8 or K18 heterozygous mice as well as WT mice of both strains survived CVB4-V virus during the 14 days studied. Two days after V-virus infection, there was a trend of elevated serum amylase and lipase in K8-null and K18-null mice compared to their respective WT and heterozygous counterparts (Table 2). The increased resistance of WT, heterozygous and K18-null mice to lethality by CVB4-V compared to published data in WT mice[21] could be due to differences in genetic background or age. Collectively, these data demonstrate that K8-null, but not K18-null, renders mice more susceptible to death by CVB4-V virus-induced pancreatitis.

Dramatic resistance and recovery from CVB4-P virus infection in K8-null mice

To determine the level of pancreatitis induced by the CVB4-viruses in the keratin-deficient mouse strains, serum amylase and lipase were determined (Table 2). Serum amylase and lipase rose in all mouse strains to a maximum 2 days after both CVB4-V and -P infection which is in close agreement with previously published data after day 3 of infection in nontransgenic mice.[22] Interestingly, K8-null mouse amylase and lipase at 2 days post infection are not as elevated after CVB4-P infection as in WT mice (Table 2). In addition, K8-null lipase levels return to normal at day 6 after CVB4-P infection while the K8 WT serum lipase were still low at day 14 due to lack of acini (Figure 2e–h). There is also a likely genetic background strain-difference between the K8 and K18 WT mice at P-2 (Table 2) despite the similar virus titer (Figure 1), which highlights the importance of using littermates as controls. The amylase and lipase levels suggest that a total lack of acinar cell keratins, as in the K8-null mice, is protective against infection by CVB4-P.

We also examined the histologic damage induced by CVB4 viruses using H&E staining. Untreated K8-null and K18-null mice have normal-appearing exocrine pancreata apart from rare areas with abnormal structures that we have described earlier[23] (Figure 2a, b, WT not shown). The severe acute/chronic pancreatitis due to CVB4-V virus 2 days post infection manifested dramatic degranulation of the acinar cells in all mice (Figure 2c, d, Table 3B). After 14 days, the survivor K8-null and all the other mice had lost their acini and the tissue was replaced by ducts, fat and inflammation (Figure 2e–h and Table 3B). K8-null survivor mice showed at day 14 a trend of higher inflammation compared to WT and K8 heterozygous mice (Table 3B). No major differences between mouse strains were observed in the hearts after CVB4-V virus (not shown).

Table 1 Survival of mice upon CVB4-V infection

Mouse genotype	Dead, %[a]	Dead/Total, n
K8 WT	0	0/9
K8+/–	0	0/10
K8–/–	40	4/10[b]
Female	20	1/5
Male	60	3/5
K18 WT	0	0/4
K18+/–	0	0/4
K18–/–	0	0/4

Notes: Mice were injected i.p. with CVB4-V virus and animal health and mortality was monitored for 14 days. The % of mice dead is shown ([a]2 of 4 K8–/– mice were found nearly dead and were sacrificed for ethical reasons). [b]Fisher's exact test shows a statistical difference between K8-null mice versus K8 WT and K8+/– mice ($p < 0.001$).

Abbreviations: WT, wild type; +/–, heterozygous; –/–, knockout; K, keratin; n, number of mice; i.p., intraperitoneal.

Table 2 Coxsackievirus induced amylase and lipase serum levels, as well as weight changes in K8 and K18 knockout mouse models

Enzyme	Mouse genotype	Control n = 4	V2 n = 3–5	V14 n = 5–10	P2 n = 4–8	P6 n = 3–4	P14 n = 3–4
Amylase,	WT	2.1 ± 0.1	15.3 ± 2.6	1.3 ± 0.1	31.9 ± 4.4	1.7 ± 0.1	2.3 ± 0.1
U/L × 10^{-3}	K8+/–	2.2 ± 0.2	18.2 ± 4.1	1.1 ± 0.9	22.1 ± 4.2	1.6 ± 0.1	2.3 ± 0.2
	K8–/–	2.9 ± 0.4	25.4 ± 6.9	1.7 ± 0.2	13.4 ± 5.5*	3.3 ± 0.0#	3.2 ± 0.2
	WT	1.6 ± 0.2	14.5 ± 9.3	1.8 ± 0.1	13.1 ± 5.3	3.2 ± 0.9	2.7 ± 0.9
	K18+/–	2.2 ± 0.1	19.9 ± 5.9	1.6 ± 0.1	6.7 ± 1.8	2.6 ± 0.3	2.1 ± 0.1
	K18–/–	2.6 ± 0.4*	24.4 ± 6.9	1.8 ± 0.3	10.5 ± 7.1	2.9 ± 0.2	2.1 ± 0.3
Lipase,	WT	90 ± 13	1108 ± 260	15 ± 1	3059 ± 480	37 ± 3	58 ± 11
U/L	K8+/–	81 ± 10	1660 ± 422	14 ± 4	1496 ± 367	37 ± 9	58 ± 6
	K8–/–	93 ± 20	2280 ± 1707	20 ± 3	924 ± 377**	103 ± 14*	123 ± 7$
	WT	134 ± 46	1793 ± 1393	19 ± 2	1767 ± 759	125 ± 16	72 ± 6
	K18+/–	194 ± 103	2762 ± 1126	23 ± 3	796 ± 162	89 ± 25	29 ± 11
	K18–/–	148 ± 39	3352 ± 1359	26 ± 4	1707 ± 1464	74 ± 5	51 ± 10
Weight difference (before–after), g	WT	0	–3.0 ± 0.7	–10.6 ± 0.9	–0.8 ± 0.3	–4.3 ± 1.5	1.3 ± 0.9
	K8+/–	–2	–2.8 ± 0.4	–9.2 ± 10	–0.6 ± 0.5	–2.0 ± 0.6	1.7 ± 0.9
	K8–/–	1	–3.0 ± 0.5	–9.1 ± 2.2	–0.7 ± 0.1	0.0 ± 1.5	1.0 ± 0.6
	WT	4	–2.3 ± 0.7	–10.8 ± 2.3	–0.3 ± 0.3	–2.0 ± 2.0	–7.3 ± 5.6
	K18+/–	1	–1.5 ± 0.6	–12.0 ± 1.2	0.3 ± 0.3	–1.3 ± 0.9	–1.8 ± 1.9
	K18–/–	3	–3.3 ± 0.8	–12.0 ± 3.9	–1.3 ± 0.9	–1.5 ± 0.9	–2.5 ± 2.6

Notes: Mice were infected i.p. with the CVB4-V or CVB4-P viruses and blood was subsequently drawn by cardiac puncture on days 2, 14 (V-virus) and days 2, 6, 14 (P-virus). Statistical differences for the K8-null mice compared to K8 WT mice was observed at CVB4-P2, -P6 and -P14 for amylase and lipase as indicated (t-test: *$p = 0.01$, **$p < 0.006$, #$p < 0.0001$, $$p < 0.01$). Values are given as means ± SEM.
Abbreviations: WT, wild type; K, keratin; n, number of mice; i.p., intraperitoneal.

The CVB4-P induced a clear destruction and degranulation of the pancreatic tissue at day 2 but the level of damage was surprisingly markedly less in K8-null mice compared to WT mice (Figure 3a, b) and K8-null acinar degranulation was significantly lower than in WT and K8+/– mice (Table 3). At day 6, the pancreas consisted of ducts, inflammatory cells and scarce acini, and no differences were observed between the mouse strains (Figure 3c, d). Analysis 14 days post-CVB4-P infection showed the recovery of the exocrine pancreas had started in all mouse strains (Figure 3e–h). Surprisingly, the K8-null mice acinar repair (Figure 3f) at this time was dramatically more complete than in WT mice (Figure 3e) with only scattered degranulation, no fat, and sometimes indistinguishable features from uninfected mice (Figure 2a). There was a similar trend for K18-null mice (Figure 3g, h) although not statistically significant (Table 3). At day 6, K8-null mice had very similar histologic manifestations of pancreatitis as WT mice (Figure 3c, d), including normalized serum enzyme levels (Table 3). This suggests that the lack of keratins associates with a milder initial damage at peak virus titers, which nevertheless leads to near complete acinar degranulation at day 6, but after that a much faster recovery.

Changing keratin patterns during CVB4-virus infection in acini and ductal cells

Keratins become hyperphosphorylated as a response to stress during many cellular events.[24] This led us to examine keratin phosphorylation and organization upon virus infection in WT and K18-null mice. The cytoplasmic acinar K8/K18 networks

Table 3A Basis for histological grading after CVB4-V and CVB4-P infection

Score	% Fat, acinar degranulation	% Inflammation
0	none	none
1	1–30	scattered
2	31–60	prominent
3	61–80	few areas with sheets
4	81–99	many areas with sheets
5	100	–

Table 3B Coxsackievirus-induced pancreatic histopathological changes in K8 and K18 knockout mouse models

Virus/Day	V2	V14		P2	P6	P14	
Feature	**Degranulation**	**Fat**	**Inflammation**	**Degranulation**	**Degranulation**	**Degranulation**	**Fat**
K8 WT	4.6 ± 0.2	2.3 ± 0.5	1.3 ± 0.2	3.3 ± 0.3	4.7 ± 0.3	4.0 ± 0.0	3.0 ± 0.0
K8+/–	4.2 ± 0.4	2.3 ± 0.3	1.5 ± 0.2	2.8 ± 0.4	4.7 ± 0.3	2.7 ± 0.7	2.3 ± 0.3
K8–/–	3.0 ± 0.7	3.4 ± 0.5	2.6 ± 0.5#	1.2 ± 0.2*	3.7 ± 0.3	1.0 ± 0.0**	0.0 ± 0.0**
K18 WT	4.3 ± 0.7	2.0 ± 0.6	1.8 ± 0.5	2.3 ± 0.0	3.0 ± 0.0	4.7 ± 0.3	3.0 ± 0.6
K18+/–	4.0 ± 0.7	2.3 ± 0.5	1.8 ± 0.3	1.3 ± 0.3	3.3 ± 0.6	3.5 ± 0.6	2.3 ± 0.6
K18–/–	4.3 ± 0.7	1.3 ± 0.3	2.0 ± 0.6	1.5 ± 0.5	4.0 ± 0.4	3.5 ± 0.9	2.3 ± 0.8

Notes: Mice were infected and harvested as described in Materials and Methods and a score of the histological criteria for fat, acinar degranulation and inflammation were given for each mouse in a "blinded" fashion as summarized in Table A. The results (B) are displayed as means ± SEM and data for +/– and null mice were compared with WT mice using Wilcoxon-Kruskal-Wallis test. CVB4-V at day 14 showed a trend of more inflammation in K8-null mice (#$p < 0.07$). CVB4-P caused less degranulation of K8-null acini at P2 (*$p < 0.001$) and P14 (**$p < 0.05$), as well as less fat (**$p < 0.05$).
Abbreviations: WT, wild type; K, keratin.

of WT cells disappeared or reorganized into clusters 2 days after CVB4-V (Figure 4a–d) and CVB4-P (Figure 5a) infection. Similarly, the AL-filaments in K18-null cells decreased (Figure 4b–e and Figure 5b). CVB4-V virus induced a dramatic proliferation of pancreatic ducts in WT, K18-null and K8-null (Figure 4g–i; ductal cells are recognized as hollow or elongated structures consisting of cells with the nucleus spanning most of the cytoplasm in contrast to the acinar cells). The lack of keratin-containing acini was also apparent (Figure 4g, h) confirming the histological data in Figure 2. CVB4-P virus induced proliferation of ducts already 6 days post-infection and the ducts were still numerous at P14 (Figure 5c–h). The regenerative nature of the CVB4-P pancreatitis induces large, new acini that in WT mice displayed extended and intricate cytoplasmic K8/K18 filaments (Figure 5e, f) and clear AL-filaments in the K18-null (Figure 5f–h).

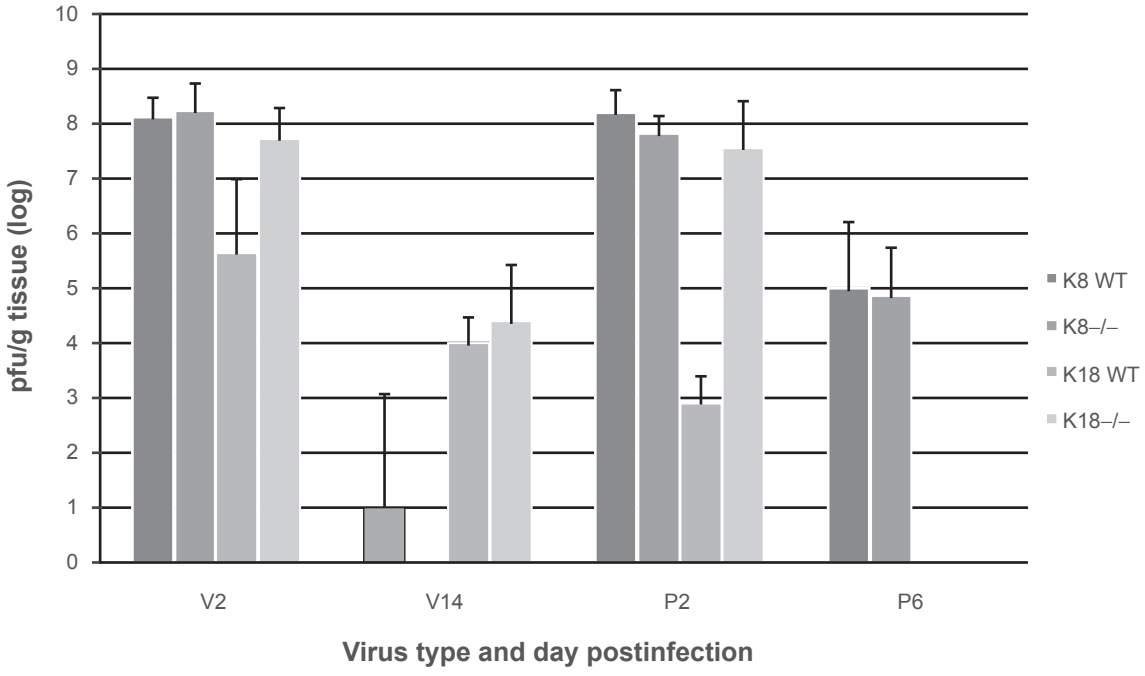

Figure 1 CVB4-V and CVB4-P can replicate normally in pancreatic tissues of keratin transgenic mice. Mice from the indicated lines (K8 WT, K8-null (–/–), K18 WT and K18-null (–/–) were infected i.p. with the CVB4-V and CVB4-P viruses and pancreata were harvested at the indicated days (V2, V14, P2, P6) after infection. The pancreatic homogenates were assayed for viral infectivity (virus titer) using a plaque assay and shown as log10 pfu/g tissue. Titers for the K18-strain at P6 were not assessed. Of note, the measured viral titer for K8-null V14 was barely detectable and this is likely because the recovered pancreatic tissue from these animals (with regenerated acini) is very small (eg, see Figure 2f). The number of mice used for each titer measurement from left to right for K8 WT, K8-null, K18 WT and K18-null were n = 5, 5, 3 and 4 for V2, n = 8, 5, 2 and 2 for V14, n = 8, 7, 4 and 3 for P2, respectively, and n = 3 K8 WT and n = 3 K8-null for P6.
Abbreviations: WT, wild type; K, keratin.

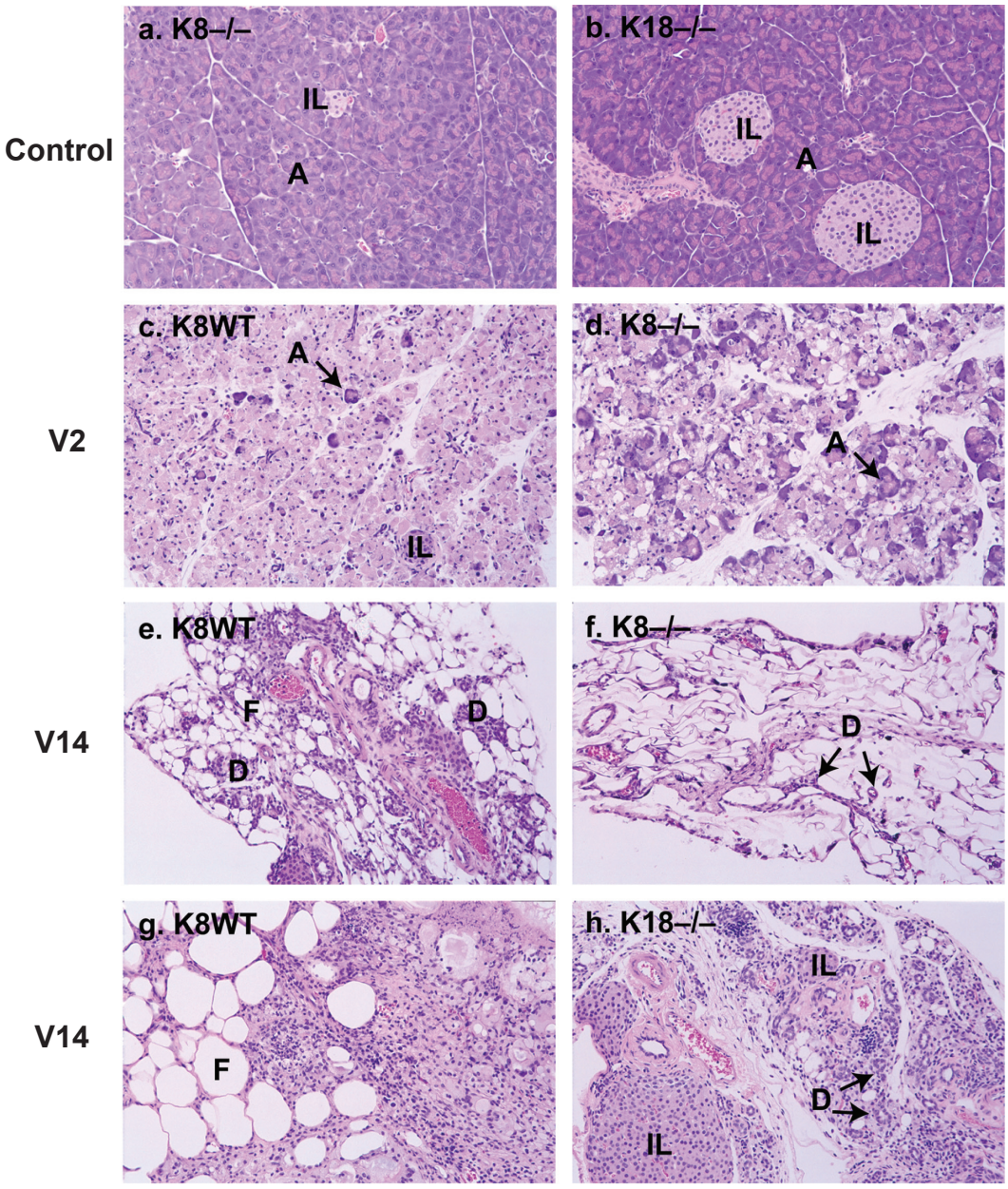

Figure 2 CVB4-V induces dramatic loss of acini in infected mice. WT and keratin-null mouse lines were injected i.p. with CVB4-V virus for 2 days (V2, c–d), 14 days (V14, e–h), or with saline (controls; a–b). Tissues were harvested, fixed and stained with hematoxylin and eosin. Lens magnification 20x.
Abbreviations: A, acini; D, ducts; F, fat; IL, islets of Langerhans; WT, wild type; K, keratin.

In order to confirm ductal regeneration and degradation of acini after CVB4 virus infection, K19 patterns were studied. K19 is the main ductal type-I keratin and a prominent marker of pancreatic ducts and displays an AL distribution in WT and K18-null mouse acinar cells as shown in Figure 6a, b.[1] The induced number of ducts (eg, days V14 and P6) stain strongly for K19 (Figure 6c–h) but no acinar K19 was detected. As expected, K19 returns to its AL domain upon recovery 14 days after P virus infection (Figure 6g, h). The lack of cytoplasmic K19 (see Figure 6) and K8 (Figure 5) in the K18-null mice upon recovery, unless missed due to the experimental time-points, suggest that this mechanism is very different from what is observed after caerulein-induced pancreatitis, where K19 forms extensive cytoplasmic filaments during the regeneration-phase that disappear after complete healing.[1]

Keratin phosphorylation is increased during CVB4-P infection

Since keratins are heavily regulated by phosphorylation especially during stress situations,[24] we investigated

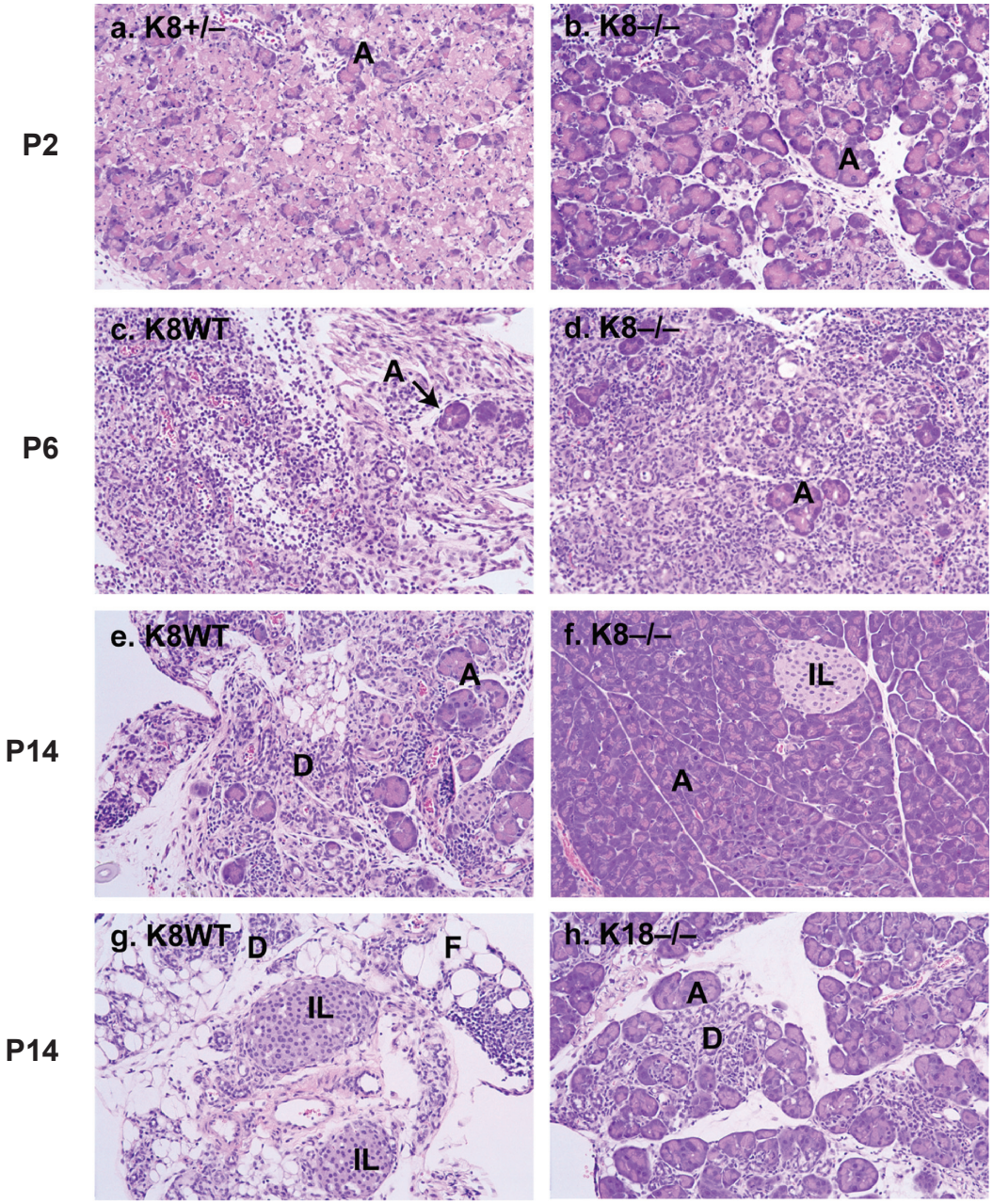

Figure 3 Less damage and faster recovery of acini in K8-null pancreas after CVB4-P induced infection. WT and keratin-null mouse strains were infected i.p. with CVB4-P virus for 2 days (P2, a–b), 6 days (P6, c–d), and 14 days (P14, e–h). Tissues were harvested, fixed and stained with hematoxylin and eosin. Lens magnification 20x.
Abbreviations: A, acini; D, ducts; F, fat; IL, islets of Langerhans; WT, wild type; K, keratin.

K8/K18 phosphorylation patterns during CVB4-P virus infection in WT mice. K18 S35 is phosphorylated in the AL acinar cell compartment during basal conditions (Figure 7Aa).[24] The phosphorylation of this site as well as K8 S438 (mitogen-stimulated)[25] are increased in apical keratin aggregates linked to bundled cytoplasmic filaments in degranulating acini during the early infection by CVB4-P at day 2 (Figure 7Aa, b). K18 S35 (which is important in filament assembly and 14-3-3-binding)[26,18] also becomes phosphorylated in proliferating ducts (Figure 7Ac), and in regenerating cytoplasmic filaments together with K8 S438 on day 14 post-infection (Figure 7Ad). The phosphorylation of the major mitosis- and stress-regulated K8 site S80[24] is increased in some duct cells (not shown) as well as in few apical areas at P14 (Figure 7Bb). When virus titers are the highest at P2, anti-CVB4 Ab stain some cells, and do partially co-localize with disrupted keratins (Figure 7Cb). Therefore, pancreatic K8 and K18 are phosphorylated in association

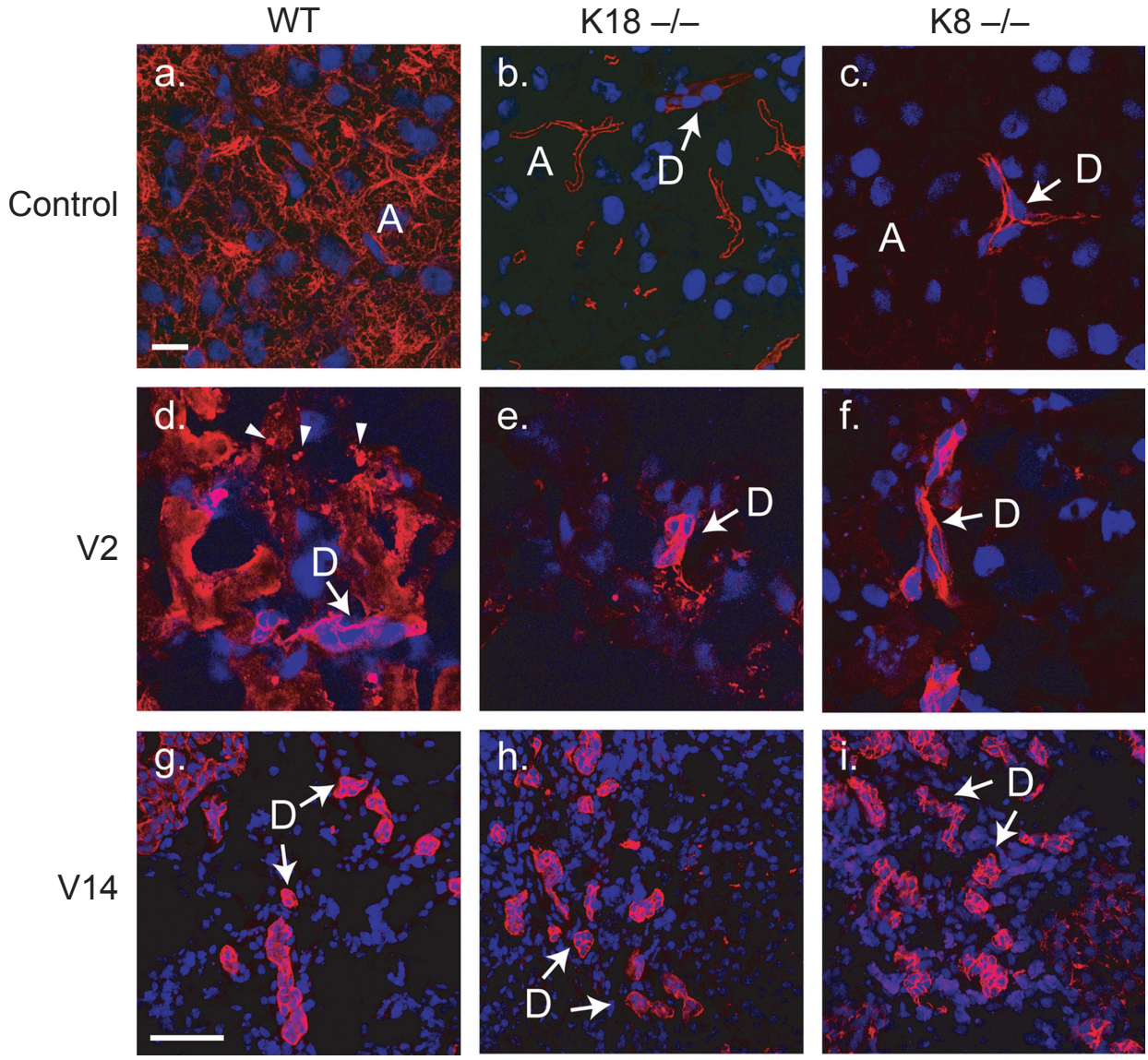

Figure 4 Keratin filament organization in WT, K8-null and K18-null mice during CVB4-V virus infection. K8 WT (a, d, g), K18 –/– (b, e, h) and K8–/– (c, f, i) mice were injected with CVB4-V (V) virus or saline (Control) and tissues were harvested at the indicated days, fresh frozen, sectioned, fixed with acetone and stained for K8/K18 (rabbit Ab 8592, shown in red) and for nuclei (Toto-1, shown in blue) arrowheads point to aggregated keratins. Scale bar in panel a (for a–f) = 10 μm, and in panel g = 10 μm (for g–i).
Abbreviations: A, acinus; D, duct; WT, wild type; K, keratin.

with CVB4-induced stress in a similar fashion to what is seen in caerulein-induced pancreatitis, and as occurs upon liver injury.[24]

Discussion

Keratins provide virus-specific protection or predisposition to pancreatitis in a virus replication-independent manner

We used K8-null pancreata as a model system of cells that lack acinar cell keratins (AL-/C-keratins) and K18-null pancreata as a model system of cells which lack only C-keratins. We demonstrated that the presence of AL-keratins is important in the outcome of the severity of pancreatitis after infection by CVB4 virus in a virus-type dependent manner (Figure 8). Total lack of acinar cell AL-/C-filaments leads to increased injury in the severe acute/chronic pancreatitis model, but less damage in the mild acute pancreatitis model. The presence of only AL-keratins, thus, is sufficient for a WT-like phenotype, while a total lack of acinar keratins is beneficial or detrimental depending on the type of virus (Figure 8). Our study is the first to show that lack of keratins in the pancreas can be beneficial by causing a less aggressive disease. This is an unusual role for any intermediate filament

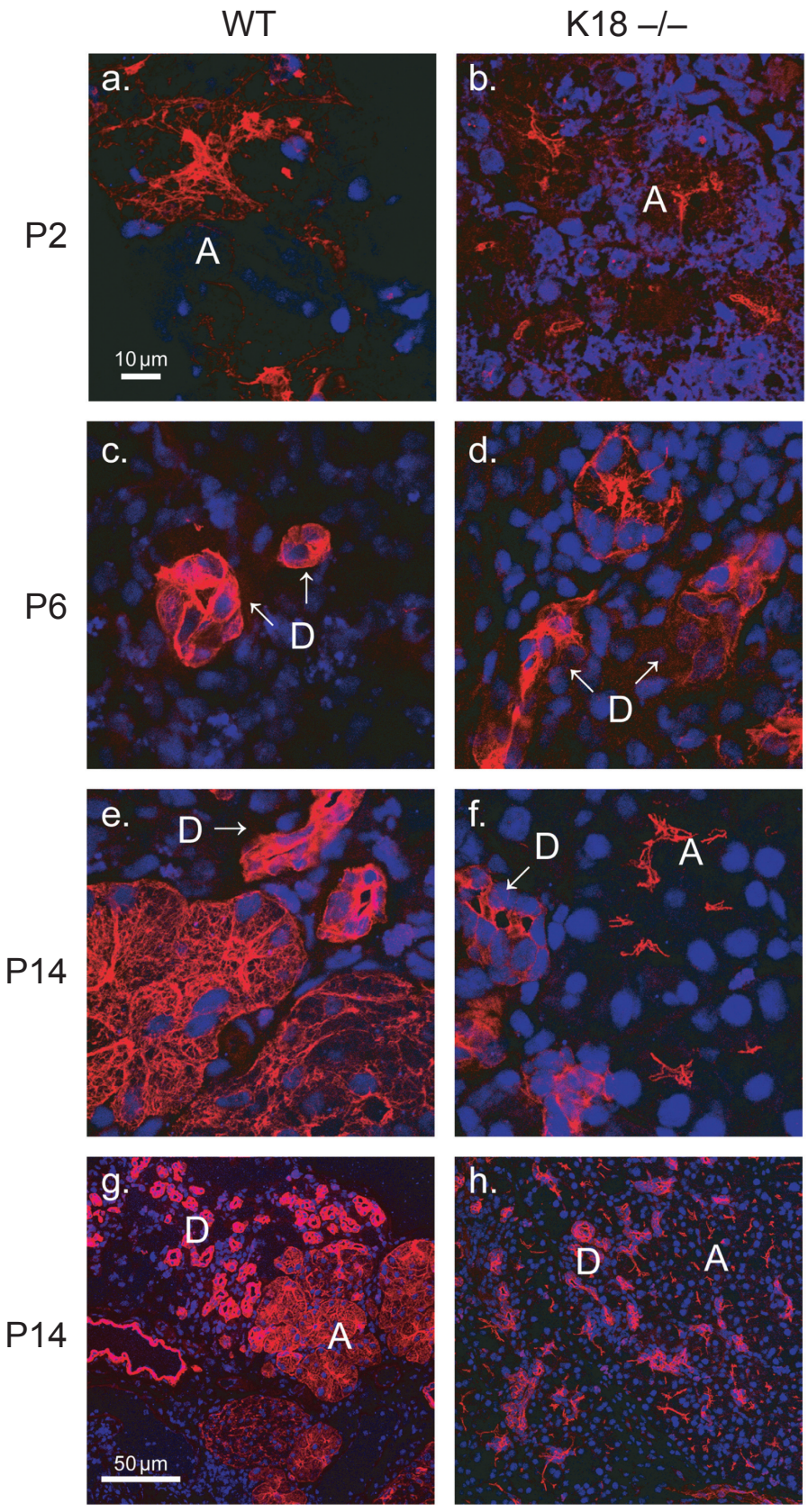

Figure 5 Keratin organization in WT and K18-null mice during CVB4-P virus infection. WT (a, c, e, g), and K18 –/– (b, d, f, h) mice were infected with CVB4-P virus and tissues were harvested at the indicated days, fresh frozen, sectioned, fixed with acetone and stained K8/K18 (rabbit Ab 8592, shown in red) and for nuclei (Toto-1, shown in blue). Scale bar in a is for a–f, scale bar in g for g, h.
Abbreviations: A, acinus; D, duct; WT, wild type; K, keratin.

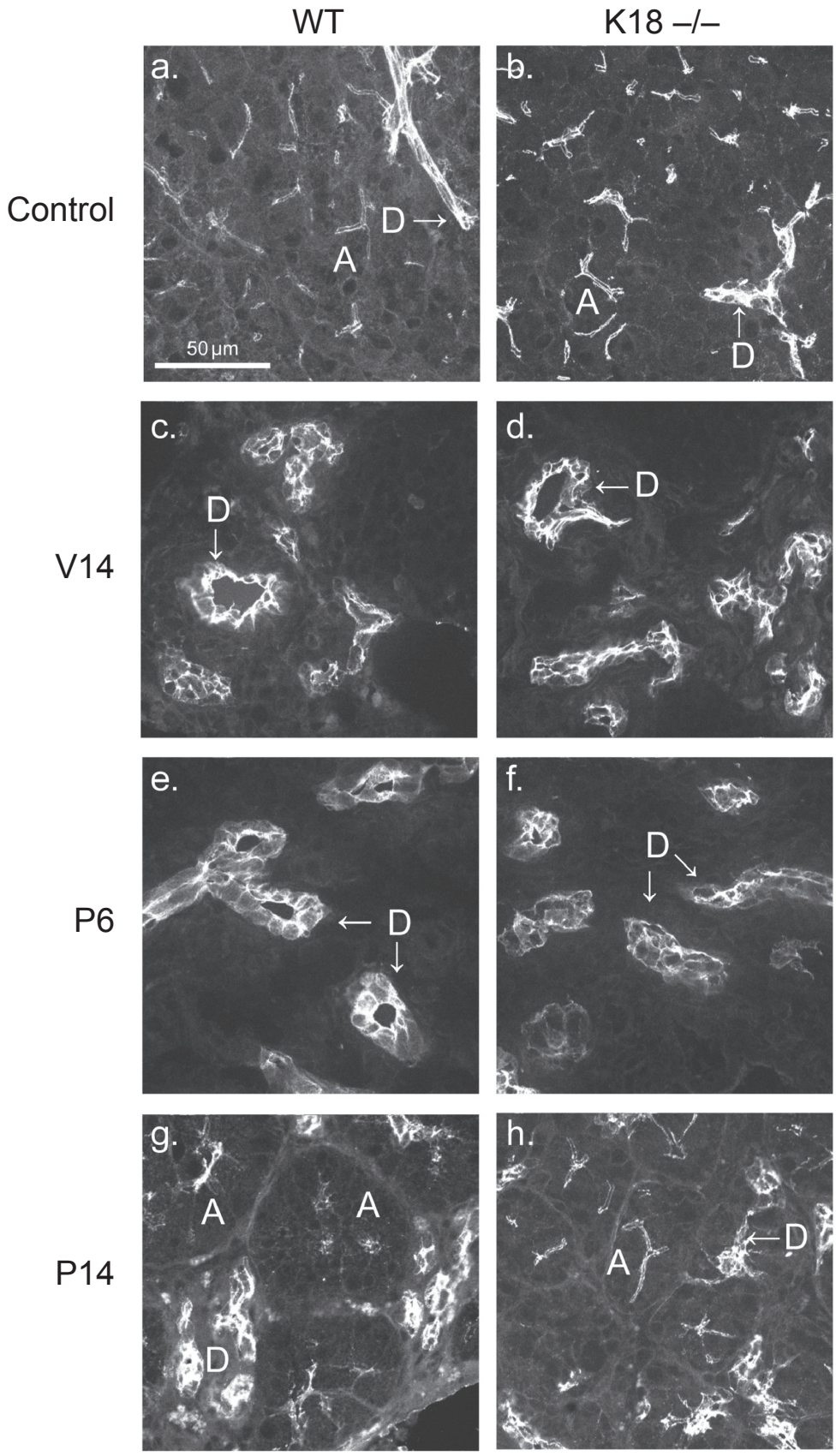

Figure 6 K19 organization in acini and ducts during CVB4-V and -P virus infections. WT (a, c, e, g) and K18–/– (b, d, f, h) mice were injected with CVB4-V (c, d), CVB4-P (e–h) or saline (a, b) and treated as in Figure 4, then stained for K19 using the rat antibody Troma III. Scale bar in a is for a–h.
Abbreviations: A, acinus; D, duct; WT, wild type; K, keratin.

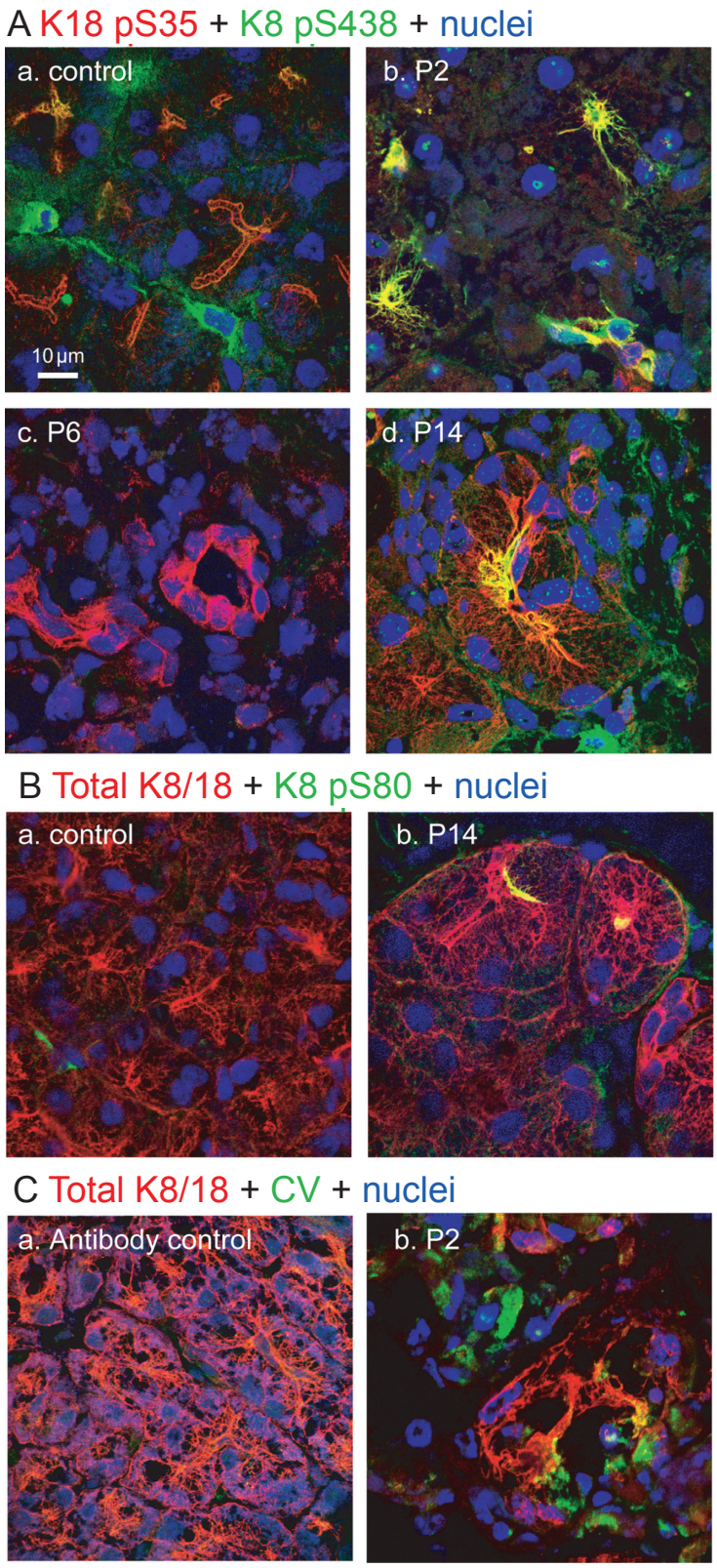

Figure 7 Keratin phosphorylation after CVB4-P infection in WT mice. Pancreata of WT mice were harvested and fixed as in Figure 4 after CVB4-P infection, then stained using anti-phospho-keratin antibodies, anti-CVB4 and nuclei (using Toto-1 shown in blue) as indicated. **A**: Control (a) and P2 (b), P6 (c) and P14 (d) time points were stained for mK18 pS35 (8250 Ab, red), mK8 pS438 (5B3 mAb, green) and nuclei (blue), **B**: Control (a) and P14 (b) samples were stained for total K8/K18 (8592 Ab, red), mK8 pS80 (LJ4 mAb, green) and nuclei (blue). **C**: WT mice 2 days after CVB4-P virus (b) were stained for K8/K18 (8592 Ab, red), coxsackievirus protein (anti-CVB4 Ab (CV), green) and nuclei (blue). In 'a' (antibody control), the anti-CV Ab did not stain untreated WT mice indicating specificity of the antibody. Colocalization of green and red is visualized as yellow. Scale bar in 'a' is for all the panels.

Abbreviations: WT, wild type; K, keratin.

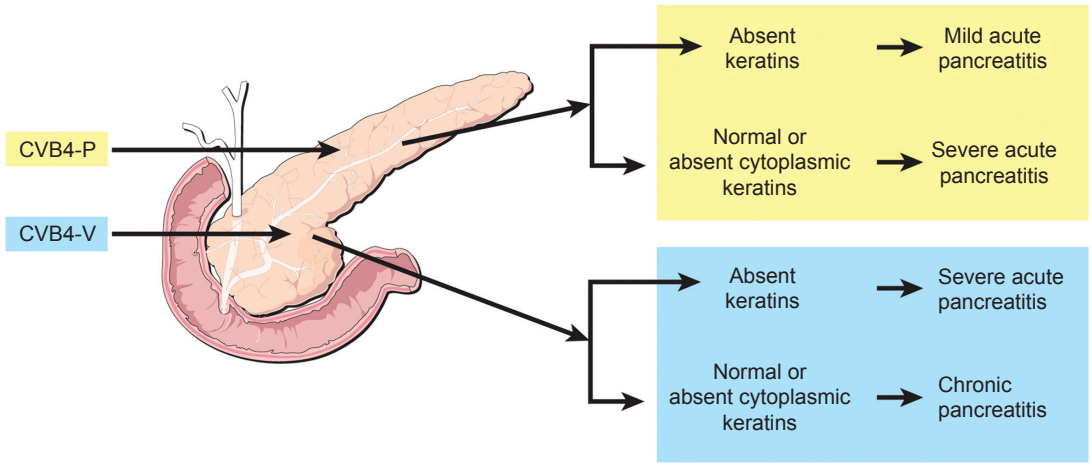

Figure 8 Schematic of the outcome of pancreatitis induced by CVB4 viruses and its dependence on the presence or absence of acinar keratins. The mouse pancreatitis induced by CVB4-P causes a milder type of acute pancreatitis, when acinar keratins are absent (K8-null), which recovers quickly compared to normal mice (WT) or mice with absent cytoplasmic keratins (but with retained apicolateral filaments; K18-null; which develop a more severe acute pancreatitis). The mouse pancreatitis induced by the CVB4-V virus is a severe acute pancreatitis, when acinar keratins are absent (K8-null), and leads to death in 40% of mice after 14 days; while mice with normal or absent cytoplasmic keratins (WT or K18-null) survive 14 days and will likely develop chronic pancreatitis.
Abbreviations: WT, wild type; K, keratin.

protein with the only other example we are aware of being the increased neurogenesis and astrogenesis that occurs in the duel absence of glial fibrillary acidic protein and vimentin.[27,28] The phenotype differences in the CVB4-infected K8-null compared to WT mice could stem from differences in virus replication; however this is likely not the case since we did not observe significant keratin genotype-related differences in virus titers. Keratin filament organization is often affected by viruses[29] and keratins are cleaved by virus proteases including those of CVB4.[30] It appears that the immunopathological effects, rather than direct virus-induced destruction, are likely responsible for the damage to acinar tissue in CVB4-induced pancreatitis.[31] However, we cannot exclude the possibility that differences in the genetic backgrounds of K18-null and K8-null mice could also contribute to the severity of infection and some of the observed findings.

Lack of acinar cell keratins guard from acute recovering pancreatitis – a contrast to the liver

The unanticipated benefit of having no acinar keratins in protection from CVB4-P-induced pancreatitis is supported by: (i) significant lower elevations in serum amylase and lipase 2 days post infection, (ii) lower histological damage at the peak of infection (day 2) and (iii) nearly complete recovery by day 14 compared to WT mice. In contrast to K8-null mice, K18-null mice did not show such protection thereby suggesting that the K8/K19-containing AL-filaments in these acini are sufficient for normal virus-induced pathogenesis. We have previously shown that K8-null, K18-null and dominant-negative mutant keratin (K18 R90C) transgenic mice exposed to two other mouse models of pancreatitis (caerulein injections and CDD diet)[1,8] does not render the mice more susceptible to pancreatitis than WT animals. If anything, K8-null mice had less inflammation after acute caerulein-induced pancreatitis as compared with their WT counterparts. The current study using the CVB4-P infection model, together with the caerulein and CDD pancreatitis models,[1,8] provide strong evidence that, in contrast to keratins in the liver, pancreatic intact acinar keratin networks are not essential for coping with acute pancreatitis.[1,8,11] The study herein takes this concept one step further by showing that total lack of acinar keratins can even be beneficial in a context-dependent manner. These conclusions mesh well with the current view that keratin mutations in humans pose a clear risk for liver disease progression while there is no apparent association with human pancreatitis (reviewed in[4,32]). For example, the early finding of an association of K8 variants with human chronic pancreatitis [33] is likely due to small patient sample size since two other much larger studies did not identify an association.[34,35]

Virus-dependent roles for keratins – possible mechanisms of action

One potential compensatory mechanism that may account for pancreas-specific functional redundancy for keratin absence is the marked up-regulation of Reg-II in K8-null pancreata.[9] Reg-II is a stress-inducible protein that is normally upregulated 1–4 days after caerulein and CDD-induced pancreatitis and is likely involved in the amelioration

of pancreatitis.[36,9] Therefore, Reg-II upregulation under basal conditions in K8-null, K18-null, and K18 R90C pancreata is a potential pro-survival candidate for the observed recovery of K8-null mice after CVB4-P infection. Reg-II is also one of the major proteins increased during the recovery period after CVB4-P infection in normal mice,[14] but not during CVB4-V infection which could explain the lack of protection or recovery from CVB4-V infection.[14] K18 is also increased after CVB4-P[14] and caerulein pancreatitis,[2] as occurs in the liver after injury[32] but Reg-II is not detected in WT or K8-null livers though other Regs are found in the liver and do serve a protective role.[39] The molecular mechanisms of Reg-II-induced regeneration and the link to keratins remain to be unraveled but may involve immune-regulation through NF-kappaB signaling.[37]

Another potential mechanism that may contribute to the pancreatitis severity differences in CVB4 infection relates to the reported effect of keratins on epithelial cell polarity and protein targeting to different subcellular compartments.[38] To this end, potential reorganization of CVB entry through the CVB receptors CAR (coxsackievirus and adenovirus receptor) and DAF (decay accelerating factor) that are located at tight junctions and that include virus-induced Abl and Fyn signaling[39] may be altered in K8-null pancreata. However, other factors are likely to be involved since viral replication after 2 days of infection appeared to be similar in all tested genotypes (Figure 1).

The role of the immune system in CVB4-V infection and possible modulation of this by keratin absence is another potential factor to consider. For example, IL-4-null mice survive CVB4-V infection, and normal mice administered IL-12 are protected from CVB4-V infection possibly due to increased IFNγ levels.[21,40] During CVB4 infection, the immunological response of a Th2-skewed pancreatic milieu during recovery co-insides with, and likely favors, acinar cell proliferation.[14] To that end, there is already evidence for keratin-related modulation of an epithelial tissue immune response as noted in K8 mouse large intestine which suffers from a Th2-type colitis,[41,38] and in K5-null mice which manifest upregulation of several cytokines in the skin.[42]

Another apparent difference is the response of K19 topographical distribution in CVB4-indued versus caerulein-induced pancreatitis. In the CVB4-induced pancreatitis, K19 was not found in WT C-filaments of recovering acini (Figure 6) as reported in the caerulein model where K19 forms temporarily extended CFs during the regeneration-phase.[1] This suggests different intracellular pathways in recovery from these two different models of pancreatitis. The above possibilities do not exclude potential caveats such as the possibility that a cytoplasmic filamentous distribution of K19 did indeed occur but was transient and could have been missed. In conclusion, our study suggests that keratins impact significantly on CVB4 virulence, positively or negatively, depending on the virus subtype and keratin makeup (Figure 8). The presence of an intact keratin filament network in pancreatic acini appears to play an important role in the outcome of experimental coxsakievirus pancreatitis which raises the possibility that keratins may serve as genetic modifiers in specific types of human pancreatitis.

Abbreviations

Ab, antibody; AL-, apicolateral; CVB4-P, Coxsackievirus B4 causing mild acute pancreatitis; CVB4-V, Coxsackievirus B4 causing lethal severe acute/chronic pancreatitis; C-, cytoplasmic filaments; h, human; htz, heterozygous; IF, intermediate filament; K, keratin; m, mouse; mAb, monoclonal antibody; pfu, plack forming unit; pS, phoshoserine, Reg-II, Regenerating islet derived-II; WT, wild type.

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Supplemental Table 1 Sex and number of mice used for CVB4-V and CVB4-P experiments

Treatment	Control (n)		V-2 (n)		V-14 (n)		P-2 (n)		P-6 (n)		P-14 (n)	
Sex	M	F	M	F	M	F	M	F	M	F	M	F
K8 WT	3	1	4	1	6	4	6	2	3	0	3	0
K8+/–	3	1	4	1	4	5	5	3	3	0	3	0
K8-null	3	1	4	1	3	5	4	3	3	0	3	0
K18 WT	2	2	2	1	1	3	4	0	3	0	2	1
K18+/–	3	1	3	1	2	2	4	0	4	0	3	1
K18-null	2	2	3	1	2	2	4	0	4	0	3	1

Notes: All mice used for CVB4-P were 2 to 3 months old and all mice used for CVB4-V were 4 months old at the time of experiment. The number of mice used for the individual measurements are indicated in the respective tables and may vary from the above table since the isolated pancreata were small and the ability to secure pieces for every determination was not possible.

Abbreviations: WT, wild type; HTZ, heterozygous; KO, knockout; K, keratin; n, number of mice.