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Overexpression of CCN1 in Het1A cells attenuates bile-induced esophageal metaplasia through suppressing non-canonical NFκB activation

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ABSTRACT

GERD is the most common gastrointestinal diagnosis given during office visit. People who suffer from a long history of GERD eventually develop Barrett's esophagus, a premalignant intestinal metaplasia due to NFκB activation. Previous studies focused on the contribution of TNF-triggered canonical NFκB pathway to this event. In this study, we demonstrated *in vitro* that it was LTA, rather than TNF, initiated canonical NFκB activation at the beginning of acid/bile attacks, but later it switched to CD40-activated non-canonical pathway, which played a bigger part in esophageal metaplasia. CCN1 attenuated this cellular transformation by suppressing CD40 and its associated proteins involved in non-canonical signaling.

1. Introduction

In response to tissue injuries or infections, our immune system jumps immediately to produce a number of cytokines like tumor necrosis factor (TNF) and chemokines like C-C motif chemokine ligand 2 (CCL2), to help the body to recover. Soon after, some transcription factors like serum response factor (SRF) [1,2] and nuclear factor kappa B (NFκB) are also activated to join the fight, leading to high expression of matrix proteins like CCN1 [3]. This is the phase of acute inflammation. If these molecules win the battle, the body returns to normal; otherwise, the inflammatory process continues and eventually turns into chronic, in which some members of the original team lose their strength and go underground, and some others even withdraw from the battle field completely. On the other hand, a group of new molecules like Lymphotoxins (LT) are recruited to the "war zone" and engage in a mild but persistent "guerrilla fight". Depending on the location of the inflammation, this phase can develop into chronic obstructive pulmonary disease (COPD), atherosclerosis, autoimmune pancreatitis, or Barrett's esophagus (BE). While chronic inflammation is a tough fight, the team can still win if the immune system is reinforced with proper medications. If they lose again, however, a cancer is on its way.

BE essentially derives from a chronic inflammation (esophagitis) caused by gastroesophageal reflux disease (GERD), in which the lower

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Abbreviations: BAFF, B cell activating factor; BAFFR, B cell activating factor receptor; BAG, BCL2 associated athanogene; BE, Barrett's esophagus; CAD, carbamoyl-phosphate synthetase; CCL, C-C motif chemokine ligand; CD27, cluster of differentiation 27; CD27L, cluster of differentiation 27 ligand; CD40, cluster of differentiation 40; CD40L, cluster of differentiation 40 ligand; CDX2, caudal type homeobox 2; CHUK, conserved helix-loop-helix ubiquitous kinase; DFFA, DNA fragmentation factor subunit alpha; EAC, esophageal adenocarcinoma; FADD, FAS-associated protein with death domain; GERD, gastroesophageal reflux disease; IAP, inhibitor of apoptosis protein; IκB, inhibitor of kappa B; IKBKB, inhibitor of nuclear factor kappa B kinase subunit beta; IKBKG, inhibitor of nuclear factor kappa B kinase subunit gamma; IKK, IκB kinase; KRT20, cytokeratin 20; LMNA, lamin A/C; LMNB, lamin B; LTA, lymphotoxin alpha; LTB, lymphotoxin beta; LTBR, lymphotoxin beta receptor; MUC2, mucin 2; NEMO, NFκB essential modulator; NFκB, nuclear factor kappa B; NIK, NFκB inducing kinase; PAK, p21 activated kinase; PARP, poly(ADP-ribose) polymerase; PRKDC, protein kinase DNA-activated catalytic polypeptide; RANK, receptor activator of NFκB; RANKL, receptor activator of NFκB ligand; RIP, receptor interacting protein; SRF, serum response factor; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; TRADD, TNFR1 associated death domain protein; TRAFF, TNF receptor associated factor; TWEAK, TNF-like weak inducer of apoptosis; TWEAKR, TNF-like weak inducer of apoptosis receptor; VIL1, villin 1

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esophageal sphincter fails to block the gastric contents to go up into the esophagus and consequently, the esophageal epithelial lining is under frequent acid attacks. Very often, the regurgitated stomach fluid contains substances like bile salts from the duodenum, which are believed to be responsible for the metaplastic transformation in BE [4–6] and also the development of esophageal adenocarcinoma (EAC) [7]. According to the literature [8], GERD patients usually have about 10 times more bile acid in their esophagus than normal people. Under the repeated insults of acidic bile, the normal squamous epithelium of the esophagus converts into a columnar phenotype like what we see in the intestine. From physiological point of view, there is nothing unusual but a natural defense mechanism of the body, because columnar epithelium is better made to endure acid erosion than squamous epithelium. However, this cellular formality change confers an increased risk of malignancy. People with BE can have as much as 400-fold higher possibility to develop esophageal cancer than the general population [9,10].

At the molecular level, BE is developed primarily due to the activation of caudal type homeobox 2 (CDX2) [11], a transcription factor that governs the intestinal specific genes like MUC2, KRT20, and VIL1. CDX2 in turn is a transcriptional target of NFκB, which is activated immediately following the massive release of inflammatory cytokines and chemokines.

Up to date, the NFκB family is composed of five individual transcription factors including NFKB1 (p105), NFKB2 (p100), RELA, RELB, and REL. Normally, these proteins are silenced in the cytoplasm by inhibitor of kappa B (IκB). Upon specific signals, NFKB1 or NFKB2 converts from their precursor form p105 and p100 into their active form p50 and p52, respectively, through canonical and non-canonical pathways correspondingly.

A variety of ligand-receptor interactions can trigger NFκB activation. The best characterized pairs are the members of TNF and TNFR families [12]. The canonical NFκB pathway is mainly activated through TNF and TNFR1 interaction in response to acute inflammation. The activated TNFR1 binds TNFR1 associated death domain protein (TRADD), which in turn recruits TNF receptor associated factor (TRAF2) and receptor interacting protein 1(RIP1). RIP1 activates IKKβ and thereby causes IκBα degradation, allowing NFKB1 to convert from p105 to p50 to transcribe genes for cell survival. In non-canonical pathway, on the other hand, only eight pairs of ligand-receptor interactions are known capable to trigger the process of NFKB2 conversion from p100 to p52, and they are LTA-TNFR2, LTA/LTB-LTBR, CD40L-CD40, RANKL-RANK, TWEAK-TWEAKR, BAFF-BAFFR, CD27L-CD27, and LIGHT-LIGHTR [13,14]. During the process non-canonical signaling, the activated receptor binds TRAF2 directly and triggers IKKα-mediated NFKB2 activation.

CCN1 is a matricellular protein that has been just recently recognized as an important component of inflammatory process [15,16]. As a secreted protein, CCN1 has the features of both conventional cytokines and extracellular matrix molecules, and therefore, it can bridge the physiological gap between structural molecules in the extracellular matrix and soluble factors like cytokines. CCN1 is expressed low in normal epithelial cells, but can be triggered to express rapidly by cytokines released during inflammation and becomes accumulated in the area, and can also stay active much longer than regular cytokines or growth factors. The elevated CCN1 helps to heal wounds, and it returns to normal level once the job is done [3]. However, if the inflammation continues overwhelmingly, CCN1 eventually disappears from the area. Consequently, a cancer development is on alert. As our previous studies showed [17,18], CCN1 was highly expressed in the esophageal epithelium of the GERD patients, then became less intense when BE developed, and once EAC occurred, CCN1 was gone almost completely. Forced overexpression of CCN1 in EAC cells repressed survivin expression and facilitated TRAIL-mediated apoptosis.

2. Materials and methods

2.1. Cell culture and transfection

Human esophageal epithelial cells Het1A (American Type Culture Collection, Manassas, USA) were maintained in culture in KBM-2 medium with supplements (Lonza, Walkersville, USA). Plasmids used for transfection were pCMV6 carrying open reading frame for human CCN1 (NM_001554) and pRS with shRNA against TNF, LTA or CD40. pRS with a negative control shRNA or pCMV6 without an insert were used as controls. All the plasmids were purchased from Origene, Rockville, USA. Cell transfections were done in Lipofectamine-2000 (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. Stable transfectants were selected using Neomycin for pCMV6 and Puromycin for pRS (Invitrogen).

2.2. Acid and bile salt treatment

To simulate GERD episodes *in vitro*, regular growth medium was adjusted to pH 4.5 with 1 N hydrochloric acid. The bile cocktail was made in reference to the literature [8,19–21], and was composed of 0.25 mM glycocholic acid, 0.10 mM sodium taurocholic acid, 0.30 mM sodium glycochenodeoxycholic acid, 0.10 mM sodium taurochenodeoxycholic acid, 0.15 mM sodium glycodeoxycholic acid, and 0.05 mM sodium taurodeoxycholic acid. All the bile salts were purchased from Sigma-Aldrich, St Louis, USA. For cell treatment, cells were plated either in Petri-dishes (for RNA or protein isolation), or on collagen-coated cover-slips (for staining). After overnight serum deprivation, cells were treated for the time and dosage as indicated. For an acute attack experiment, cells were cultured in the conditioned medium for 15 min, and then RNA and protein were isolated either immediately or after 2-hour recovery in the regular growth medium. To simulate chronic acid reflux, cells were briefly rinsed with the conditioned medium, 4 times a day with a 2-hour interval of resting in the growth medium at 37 °C. The episodes were repeated daily for a week.

2.3. Immunofluorescence and immunocytochemistry

Cells were cultured till desired confluence on coverslips that had been pre-coated with type I collagen. After serum starvation and indicated treatment, cells were fixed for 10 min in 4% paraformaldehyde. For immunofluorescence, after incubation with a primary antibody for 2 h, cells were washed in PBS and incubated with a FITC- or Texas Red-conjugated secondary antibody (Abcam, Cambridge, USA) for an hour. Nuclei were counter-stained with Propidium Iodide or DAPI (Invitrogen). For immunocytochemistry, instead of fluorescent-conjugated secondary antibodies, cells were incubated with the LSAB + kit (DAKO, Carpentaria, USA) containing a universal secondary linker and HRP-conjugated streptavidin with AEC chromogen as substrate. Nuclei were counter-stained with hematoxylin. The following antibodies were used in cell staining: MUC2, KRT4, KRT20, VIL1, and CDX2 (Abcam); NFKB1, NFKB2, CD40, and LTBR (Origene).

2.4. Real-Time RT-PCR analysis

As described in our previous publications [3,22], total RNA was extracted using RNeasy kit (Qiagen, Valencia, USA) following the manufacturer's protocol. Reverse transcription was done in MyCycler (Bio-Rad, Hercules, USA) following the procedure: 25 °C/10 min, 55 °C/30 min, 85 °C/5 min, and 4 °C/∞. All of the reagents for reverse transcription, including Reverse Transcriptase, Nucleotide Mix, Protector RNase Inhibitor, and Random Primer $p[dN]_6$, were purchased from Roche (Mannheim, Germany). Real-time PCR was performed in iCycler (Bio-Rad) following the two-step program, using SYBR Green master mix (SABiosciences, Frederick, USA). A 96-well gene array containing all the known members of TNF and TNFR families as well as the genes involved in their signaling was used to examine the overall effect of acid/bile treatment. The array and the following primers were purchased from Origene:

GGAAAAGGCAGCTCACTGAAGC/GGAGATACCAGTTCCACAGGTC (CCN1, NM_001554);

ACTCTCCACACCCAGCATCATC/GTGTCCCGTATGTGCCGTTGT (MUC2, NM_002457);

GCTGCTCTACACCTACCTCATC/TTCTGGTCCAGGATGACGGCTT (VIL1, NM_007127);

GGATTCGAGGTTCAAGTCACGG/TCTAGGTTGCGCTCCAGAGACT (KRT20, NM_023256);

GCCGAGAATGACTTGTGGTCC/CTCCGCATCATAGAGGACCTTC (KRT4, NM_002272);

ACAGTCGCTACATCACCATCCG/CCTCTCCTTGCTCTGCGGTTC (CDX2, NM_001265);

CCGCTTCAGAAAACCACCTCAG/ATGCCGGTACTGGTTCTTCCTG (TNFR1, NM_001065);

CGTTCTCCAACACGACTTCATCC/ACGTGCAGACTGCATCCATGCT (TNFR2, NM_001066);

CAACGAGCACTGGAACTACCTG/GGTCTTCCGTTTGCTTGTGCAG (LTBR, NM_002342);

GCAGCACTACTTCTTGACCACC/TCTGCTCCTGAGCATTGACGTC (NFKB1, NM_003998);

GGCAGACCAGTGTCATTGAGCA/CAGCAGAAAGCTCACCACACTC (NFKB2, NM_002502); and

GTCTCCTCTGACTTCAACAGCG/ACCACCCTGTTGCTGTAGCCAA (GAPDH).

Data were generated from at least 5 independent experiments and analyzed according to the $\Delta\Delta C_t$ method. Briefly, ΔC_t was calculated by subtracting the C_t value of GAPDH from the C_t value for each gene; and then $\Delta\Delta C_t$ was calculated by subtracting the ΔC_t of the control from the ΔC_t of the treatment; and finally the fold change was calculated using the formula: Fold Change = $2^{(-\Delta\Delta Ct)}$.

2.5. Western blot analysis

The following antibodies were used in Western blot analysis: CCN1 (Santa Cruz Biotechnology, Santa Cruz, USA); MUC2, KRT4, KRT20, VIL1, CDX2, and GAPDH (Abcam); NFKB1, NFKB2, CD40, and LTBR (Origene). Detailed procedure can be found in our previous publications [1–3]. Signals were quantified with the TotalLab TL100 software (Nonlinear Dynamics, Garth Heads, UK) based on at least five replicates.

2.6. Statistical analysis

All numerical data were analyzed by single classification one-way ANOVA and $P < 0.05$ was considered significant.

3. Results

3.1. Acid rather than bile exposure upregulates CCN1 expression in esophageal epithelial cells

According to clinical data [23], 60% of GERD episodes bring the acidity in the lower esophagus down to pH 4.0–7.0. However, GERD is not always a reflux of pure gastric acid but rather periodic regurgitations of a mixture of gastric and duodenal contents [8,19–21]. The latter is enriched of bile salts, which are believed to be the cause of esophageal metaplasia and adenocarcinoma [7]. Based on our previous study, which demonstrated a heavy expression of CCN1 in the esophageal epithelium of GERD patients [17], we wanted to know the real cause of this upregulation, acid or bile. So we cultured esophageal epithelial cells (Het1A) for a week in one of the following conditions: (1) complete medium of pH 4.5, (2) complete medium containing bile salts at pH 7.0, (3) complete medium containing bile salts at pH 4.5, and (4) regular growth medium (control). RNA and protein were isolated to assess CCN1 expression at the time of 30 min, 6 h, 24 h and 7 days. CCN1 was found highly upregulated in a time-dependent manner in response to acid treatment, and its mRNA level was up by 80 fold ($P < 0.01$) after one week of acid exposure. In bile salts, on the other hand, CCN1 actually went down a bit (Fig. 1A and B). When the cells were given a combination of acid and bile, CCN1 expression was still elevated by approximately 5-fold ($P < 0.05$), compared to its normal level, but much less than it in acid alone, suggesting that, between acid and bile, pH drop was the reason for the overexpression of CCN1 in the esophageal epithelium of GERD patients.

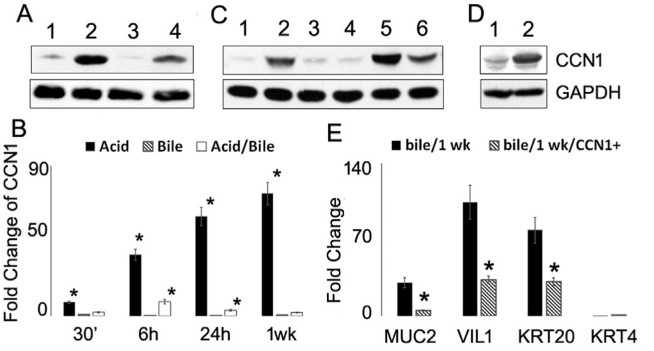

Fig. 1. CCN1 expression in response to acid, bile, or acid/bile in Het1A cells. A. Western blot analysis of CCN1 protein expression in response to 6-hour acid or bile or acid/bile treatment. Lanes: 1. Control, 2. acidic (pH 4.5), 3. Neutral bile (pH 7.0), 4. Acidic bile (pH 4.5). B. Real time RT_PCR analysis of CCN1 gene expression in response to acid, bile or acid/bile over the time course of 30 min, 6 h, 24 h and 1 week. The numbers represent fold change against the corresponding control (regular growth medium). Numerical data are presented as mean ± standard deviation. *Statistical significant ($P < 0.01$). C. Western blot analysis of CCN1 protein expression in response to acute or chronic acid attacks. Lanes: 1. Control (15 min), 2. 15-min acute acid attack, 3. 15-min acute acid attack followed by 2-hour recovery, 4. Control (1 week), 5. 1-week chronic acid, 6. 1-week chronic acid followed by 2-hour recovery. D. Western blot analysis verifying CCN1 transfection. Lane 1, Het1A cells transfected with pCMV6 vector; lane 2, Het1A cells transfected with pCMV6-CCN1 plasmids. E. Real time RT_PCR analyses showing the effect of CCN1 overexpression on bile salts-induced esophageal metaplasia. Control Het1A and Het1A^{CCN1+} cells were cultured in bile salts containing medium for the time indicated. RNA was isolated to assess the expressions of MUC2, VIL1, KRT20 and KRT4. The numbers represent fold change against the corresponding control (regular growth medium). Numerical data are presented as mean ± standard deviation. *Statistical significant ($P < 0.01$).

GERD, as we know, is not a constant but episodic exposure of esophageal cells to acidic fluid. In order to simulate the clinical condition of GERD *in vitro* more closely, we ran two types of experiments: acute and chronic. The former was just a 15-min acid exposure (pH 4.5), while the latter was done by 4 flushes with the acidic medium per day, with a 2-hour interval in regular growth medium, for a course of 7 days. CCN1 was found significantly elevated in response to both acute and chronic acid exposure (Fig. 1C). After an acute acid attack, CCN1 level went up sharply, then came down to normal after 2-hour recovery in regular growth medium. Following a week of episodes of chronic acid exposure, however, CCN1 stayed at a fairly high level even after the recovery.

3.2. Overexpression of CCN1 increases cell resistance to bile-induced esophageal metaplasia

When GERD episodes continue for a long period of time, there is a great chance to develop BE, a premalignant condition in which stratified esophageal squamous epithelium is replaced by simple intestinal columnar epithelium. Bile salts in acid reflux are believed to be the cause [5]. According to our previous study [17], CCN1 expression in the

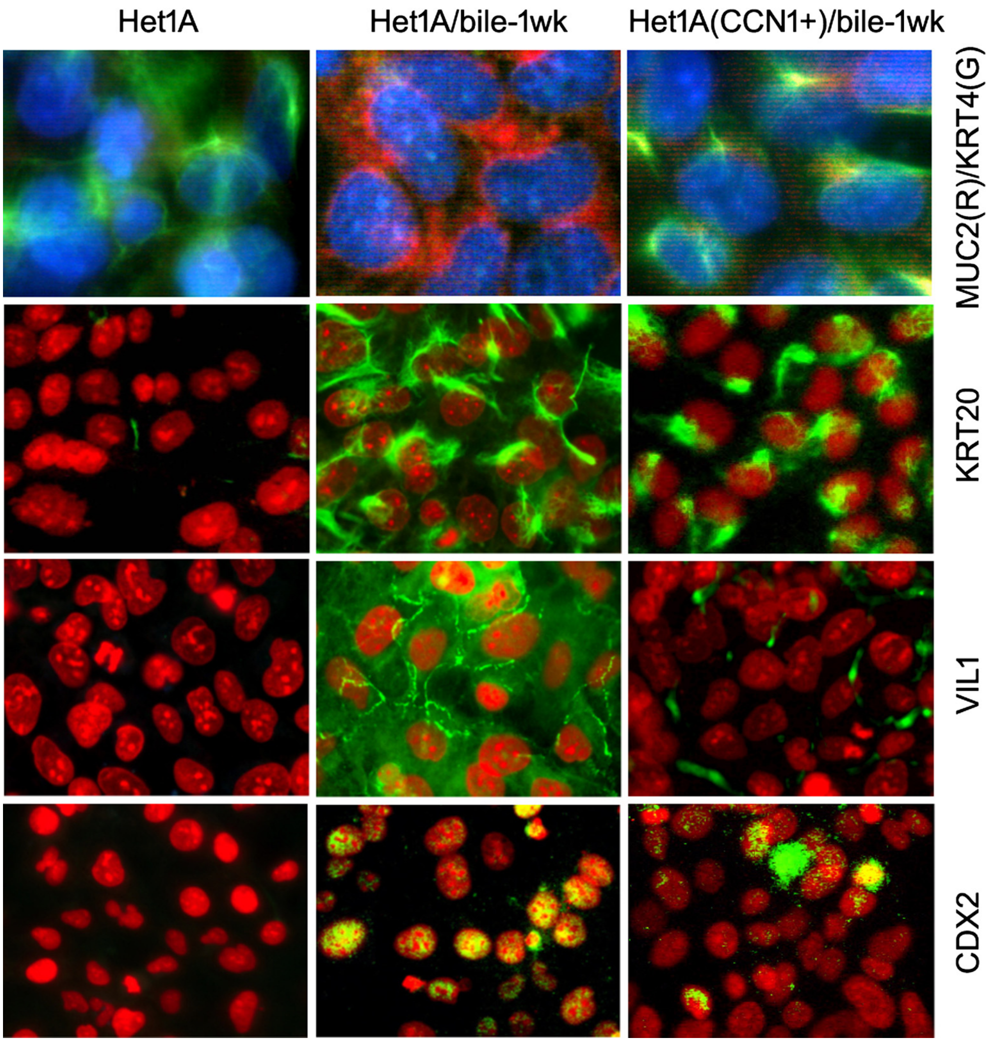

Fig. 2. Immunofluorescence cell staining showing the effect of CCN1 overexpression in Het1A cells on bile-induced expression of intestinal makers. Cells were cultured in bile salts containing medium for a week. Top panel: double staining using mouse anti-MUC2 and rabbit anti-cytokeratin 4 primary antibody, and developing with Texas Red-conjugated goat anti-mouse and FITC-conjugated goat anti-rabbit secondary antibody. Nuclei were counter-stained with DAPI. Second panel: staining using rabbit anti-cytokeratin 20 primary antibody and FITC-conjugated goat anti-rabbit secondary antibody. Nuclei were counter-stained with propidium iodide. Third panel: staining using rabbit anti-villin primary antibody and FITC-conjugated goat anti-rabbit secondary antibody. Nuclei were counter-stained with propidium iodide. Fourth panel: staining using rabbit anti-CDX2 primary antibody and FITC-conjugated goat anti-rabbit secondary antibody. Nuclei were counter-stained with propidium iodide.

transformed cells was still higher than normal, but much less than it in the early phase of GERD. We wanted to know the role of CCN1 in the development of BE. So we transfected Het1A cells with a plasmid carrying CCN1 open reading frame (Fig. 1D), and then subjected the cells to one-week chronic bile treatment. Expressions of intestinal epithelial markers including MUC2, VIL1, KRT20 and CDX2 along with the esophageal epithelial marker KRT4 were examined.

Based on RT-PCR analysis, chronic bile exposure activated expressions of MUC2, VIL1 and KRT20 in esophageal epithelial cells but downregulated KRT4 (Fig. 1E). Constitutional overexpression of CCN1 increased the cellular resistance to this type of metaplastic change, namely, it lowered bile-induced expressions of MUC2 by 82%, VIL1 by 68%, and KRT20 by 60% (all $P < 0.05$), compared to the cells transfected with the empty vector. The expression of KRT4 in Het1A^{CCN1+} cells remained almost unchanged after a week of bile treatment. These results were also supported by immunofluorescence cell staining (Fig. 2), which also demonstrated that nuclear expression of CDX2 in the presence of bile salts was nearly gone due to CCN1 overexpression.

3.3. Activation of NFκB in response to chronic acid/bile insults is mainly attributed to non-canonical signaling

As mentioned above, most of the intestinal markers are transcriptionally controlled by CDX2, a transcription factor that is not expressed in normal esophageal epithelium but can be activated to express by acid/bile stimuli via NFκB regulation [11]. Previous studies mostly focused on p105 to p50 conversion in response to acid/bile treatment [24,25], implying that NFκB was activated through the canonical pathway during esophageal metaplasia. However, based on our data derived from Western blot analyses and immunocytochemistry, this might not be the entire case. Although p50 expression and its nuclear localization were detected in our experiment, NFKB1 expression level stayed relatively low during the whole week of acid/bile treatment (Fig. 3). NFKB2, on the contrary, was highly expressed and also heavily localized to the nucleus in response to chronic acid/bile insults. Moreover, Western blot analysis demonstrated a prominent conversion of p52, compared to NFKB1/p50. Overexpression of CCN1 clearly lowered p52 nuclear translocation, while p50 remained almost unaffected (Fig. 3).

3.4. Chronic acid/bile exposure upregulates the core elements of non-canonical NFκB signaling

As discussed earlier, TNF family cytokines along with their receptors are the best known activators of both canonical and non-canonical NFκB pathways [12]. We wondered how acid/bile exposure affected these two super-families in esophageal epithelial cells. So we used the RNA extracts generated from the chronic acid/bile experiment to probe a gene array containing all the members of TNF and TNFR family, as well as the molecules involved in their signaling. As shown in Table 1, the top upregulated genes in response to chronic acid/bile exposure predominantly fell in two categories: cell survival/death and NFκB activation/inhibition. The first category included three pro-apoptotic genes, DFFA, CASP8, and CASP2, and the rest were pro-survivals,

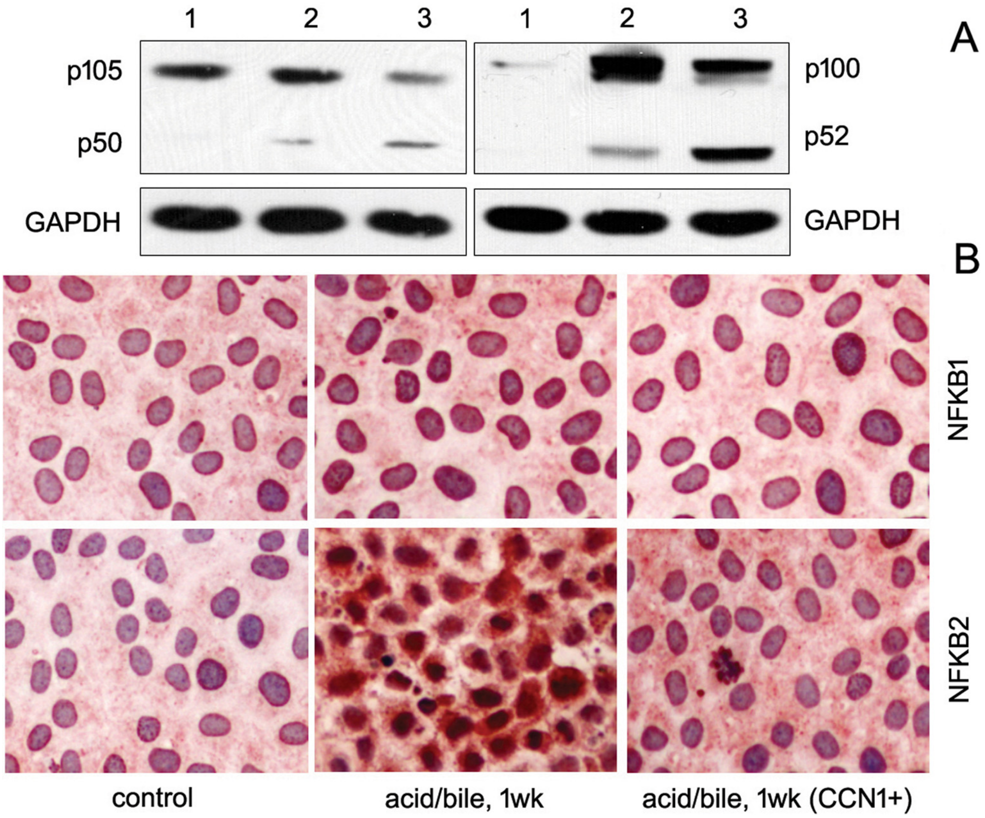

Fig. 3. Overexpression of CCN1 suppresses chronic acid/bile-activated NFKB2 expression and nuclear translocation. Control Het1A and Het1A^{CCN1+} cells were given chronic acid/bile treatment for a week, in petri dishes as well as on coverslips. Protein was isolated from the petri dishes for Western blot analysis, while the coverslips were processed for immunocytochemistry. A. lane 1 – control, lane 2 – acid/bile treatment for 24 h, lane 3 – acid/bile treatment for a week. B. Cells were fixed in 3.7% formaldehyde, permeabilized in cold acetone, incubated with the primary antibody as indicated, and then stained with a HRP-conjugated secondary antibody. Color was developed in AEC chromogen. Hematoxylin was used for counterstaining.

Table 1

Top upregulated genes (in descending order) in Het1A cells in response to chronic acid/bile exposure. Data derived from gene array analysis of TNF/TNFR families and associated molecules. The numbers represent fold changes against control. > 2 fold was considered as significant.

Genes	Acid/bile, 1 week	Functions
LMNB2	75281.10	DNA repair
PARP1	75281.10	DNA repair
PRKDC	75281.10	DNA repair
CD40 (TNFRSF5)	61147.25	Non-canonical activation of NFκB
HSPB1	61147.25	Stress response
SUMO1	61147.25	Nuclear transport
TRADD	57052.40	Canonical activation of NFκB
LTBR (TNFRSF3)	53231.77	Non-canonical activation of NFκB
DFFA	40342.14	Apoptosis
RNF216	24833.50	Inhibition of NFκB activation
PRKACA	24833.50	Supporting NFκB
LMNA	23170.48	DNA repair
PSMG2	21618.82	Proteasome assembly
CAD	20171.07	DNA synthesis
CHUK (IKKα)	15286.81	Non-canonical activation of NFκB
IKBKAP	15286.81	NIK-IKK assembly
JUN	14263.10	Cell proliferation
PAK1	14263.10	Cell survival
TANK	11585.24	TRAF inhibitor
BAG4	8192.00	Apoptosis inhibition
NFKB2	7749.21	Precursor of non-canonical NFκB
TRAF2	5404.70	NFκB activation
CASP8	4096.00	Apoptosis
CASP2	3821.70	Apoptosis
TNFAIP3 (A20)	3104.19	Inhibition of canonical NFκB activation
IKBKB (IKKβ)	2702.35	Canonical NFκB activation
RANK (TNFRSF11A)	1552.09	Non-canonical activation of NFκB
BAFF (TNFSF13B)	1176.27	Non-canonical activation of NFκB
TNFSF15	315.17	NFκB activation
CD40L (TNFSF5)	238.86	Non-canonical activation of NFκB
LTA (TNFSF1)	73.52	Non-canonical activation of NFκB

Table 2

The early response of NFκB network to acid/bile exposure in Het1A cells. Data derived from gene array analyses of TNF/TNFR families and associated molecules. Numbers represent fold changes against their corresponding controls. > 2 fold was considered as significant.

Genes	Acid/bile, 30 min	Acid/bile, 2 h	Acid/bile, 1 week
TNF (TNFSF2)	0.66	0	0.54
TNFR1 (TNFRSF1A)	68.06	0.44	0.50
TNFR2 (TNFRSF1B)	0.87	1825676.88	0.50
TRADD	132.89	4.90	57052.06
CHUK (IKKα)	0.93	3.10	15286.00
IKBKB (IKKβ)	6225.25	2.64	2702.85
IKBKG (NEMO)	1.74	0.47	0
NFKB1	93	18.44	0.50
NFKB2	0.78	15.55	7749.21
JUN (AP-1)	3.03	15286.90	14263.22
LTA (TNFSF1)	32768.41	294.47	73.46
LTB (TNFSF3)	1.25	0.66	0.62
LTBR (TNFRSF3)	0.75	0.44	53231.55
CD40L (TNFSF5)	0.22	0.72	238.86
CD40 (TNFRSF5)	1.37	4705.44	61147.23
RANKL (TNFSF11)	0.47	0.01	0.01
RANK (TNFRSF11A)	1.22	0.88	1552.90
TRAF1	15.75	0.18	0.62
TRAF2	3.08	11.49	5404.36
TRAF3	5.50	0.18	0.57
AGFG1 (RIP1)	3.12	0	0.87
BIRC3 (CIAP2)	2.44	1.32	0.50
TNFAIP3 (A20)	2.67	2896.08	3104.23

including LMNB2, LMNA, PARP1, PRKDC, SUMO1, CAD, PAK1, and BAG4, suggesting that cells were actively fighting against the harsh condition rather than simply surrendering to it. In the second class, most were supporters for NFκB activation, especially those involving in non-canonical signaling, such as the receptor genes like LTBR, CD40 and RANK, ligand genes like CD40L, BAFF and LTA, NIK-associated molecules like IKBKAP, and more importantly, CHUK (IKKα), the

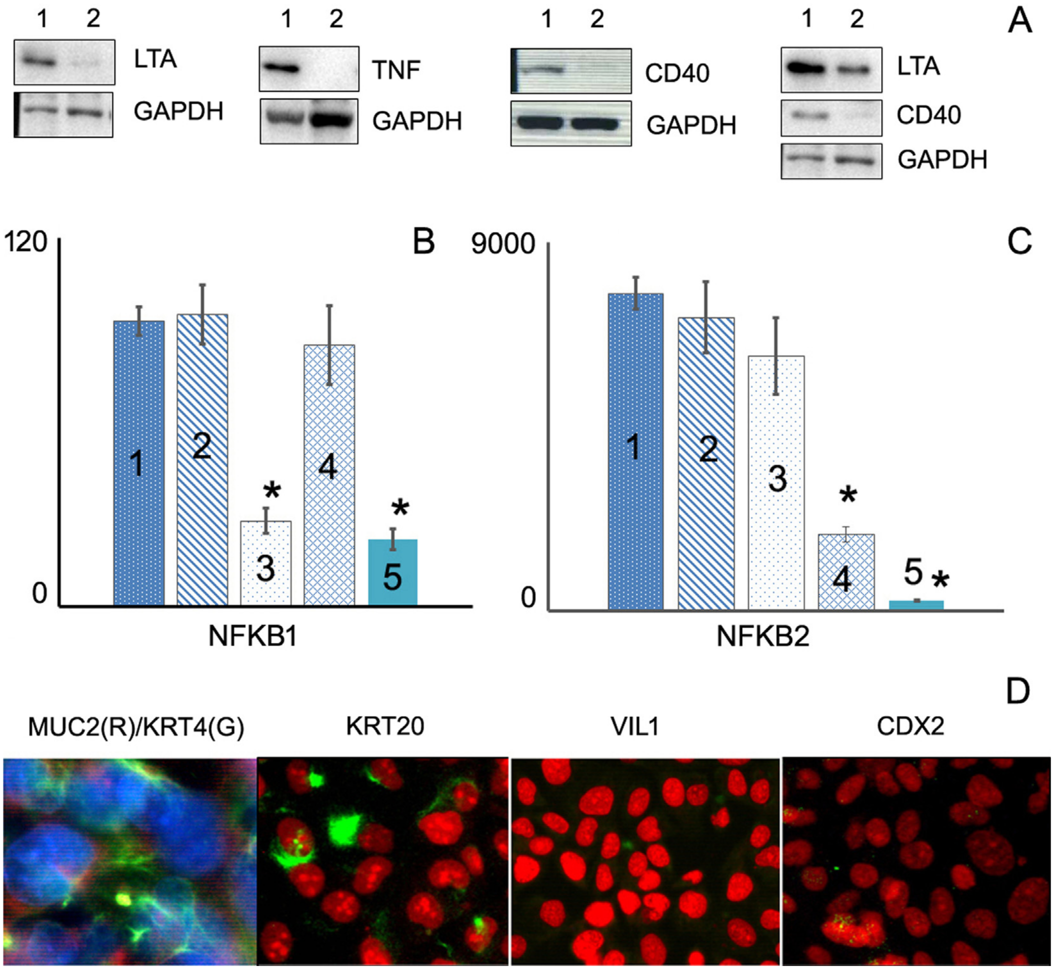

Fig. 4. The roles of TNF, LTA and CD40 in acid/bile-induced NFκB activation. A. Western blot analyses verifying gene knockdown efficiencies (2) against negative control (1) in Het1A cells. From left to right: LTA knockdown, TNF knockdown, CD40 knockdown, LTA and CD40 double knockdown. B. RT_PCR analyses showing the effects of TNF, LTA or CD40 knockdown on NFKB1 expression after 30 min acid/bile exposure: 1 – control (negative sequence transfection), 2 – TNF knockdown, 3 – LTA knockdown, 4 – CD40 knockdown, 5 – LTA and CD40 double knockdown. Numerical data are expressed as mean ± standard deviation. *Statistical significant ($P < 0.01$). C. RT_PCR analyses showing the effects of TNF, LTA or CD40 knockdown on NFKB2 expression after one week acid/bile exposure: 1 – control (negative sequence transfection), 2 – TNF knockdown, 3 – LTA knockdown, 4 – CD40 knockdown, 5 – LTA and CD40 double knockdown. Numerical data are presented as mean ± standard deviation. *Statistical significant ($P < 0.01$). D. Immunofluorescence staining showing the effect of CD40 knockdown on bile-triggered expression of intestinal markers. From left to right, 1st panel: double staining using mouse anti-MUC2 and rabbit anti-cytokeratin 4 primary antibody, and developing with Texas Red-conjugated goat anti-mouse and FITC-conjugated goat anti-rabbit secondary antibody. Nuclei were counter-stained with DAPI. 2nd panel: staining using rabbit anti-cytokeratin 20 primary antibody and FITC-conjugated goat anti-rabbit secondary antibody. Nuclei were counter-stained with propidium iodide. 3rd panel: staining using rabbit anti-villin primary antibody and FITC-conjugated goat anti-rabbit secondary antibody. Nuclei were counter-stained with propidium iodide. 4th panel: staining using rabbit anti-CDX2 primary antibody and FITC-conjugated goat anti-rabbit secondary antibody. Nuclei were counter-stained with propidium iodide.

ultimate trigger for converting NFKB2/p100 to p52. TRADD and IKBKB (IKKβ), the two important components for canonical signaling were also among the top of the list, suggesting that both canonical and non-canonical pathways participated in the response to acid/bile treatment but the latter might have carried on more load of work. On the other hand, 3 inhibitors of NFκB were also triggered to express, including RNF216, TNFAIP3 (A20) and TANK (Table 1). The first two are inhibitors specifically for the canonical activation of NFκB, while TANK inhibits NFκB activation by eliminating TRAF1/2/3.

3.5. *LTA activates NFκB via canonical first and non-canonical later during the course of chronic acid/bile exposure*

As shown in Fig. 3 and Table 1, although NFKB1 stayed relatively low compared to NFKB2 during the course of acid/bile exposure, p50 expression was detectable at both 24 h and 1 week of the treatment. We wondered how NFKB1 activation took place. So we looked at the expression of these genes at earlier time points, namely, 30 min and 2 h in acid/bile-conditioned medium. To our surprise, at 30 min most of the upregulated genes were those related to canonical NFκB signaling (Table 2), including TNFR1, TRADD, IKBKB, and NFKB1. However, TNF, the best-known NFKB1 activator, did not respond to the treatment; instead, LTA was found highly expressed from the very beginning, and also stayed in elevation through the entire week of the acid/bile treatment, although it became weaker toward the end of the experiment. At 2 h, on the other hand, TNFR2 showed a robust expression to replace TNFR1.

We know that LTA can form homo-trimers, like TNF, to trigger NFKB1 activation by binding to TNFR1, or TNFR2 when it is available, the latter can activate both NFKB1 and NFKB2 [12]. In addition, LTA is also capable to form hetero-trimers with LTB to signal through receptor LTBR particularly to trigger the non-canonical pathway [26,27]. Based on our experiment, these three receptors were activated in such order: TNFR1 at 30 min, TNFR2 at 2 h, and LTBR at one week during the acid/

Table 3
The effect of CCN1 overexpression on the genes involved in non-canonical activation of NFκB. Data derived from gene array analyses of TNF/TNFR families and associated molecules. The values represent fold changes against the corresponding control. > 2 fold was considered as significant.

Common name	Gene name	Acid/bile/Het1A, 1 week	Acid/bile/Het1A^{CCN1+}, 1 week
LTA	TNFSF1	73.50 ± 13	46.00 ± 18
LTB	TNFSF3	0.62	3.73
LTBR	TNFRSF3	53231 ± 55	0
CD40L	TNFSF5	238 ± 12	1.23
CD40	TNFRSF5	61147 ± 41	2.46
RANKL	TNFSF11	0.01	1.23
RANK	TNFRSF11A	1552 ± 44	1274 ± 29
TWEAK	TNFSF12	0.71	0.62
TWEAKR	TNFRSF12A	0.41	0.57
BAFF	TNFSF13B	1176 ± 55	1362 ± 47
BAFFR	TNFRSF13C	0	0.50
LIGHT	TNFSF14	0.50	2.30
LIGHTR	TNFRSF14	0.27	1.23
CD27L	TNFSF7	0.78	1.15
CD27	TNFRSF7	0	1.23
TRAF2	TRAF2	5404 ± 81	0
TRAF3	TRAF3	0.57	1.23
CIAP1	BIRC2	1.52	0.57
CIAP2	BIRC3	0.50	0
IKKα	CHUK	15286 ± 112	1.23

bile treatment, so we thought that LTA might activate NFKB1 first and NFKB2 later. To test this hypothesis, we used a shRNA vector to knock down LTA, or TNF as a reference, in Het1A cells (Fig. 4A), and then repeated the experiment. Knockdown of LTA suppressed acid/bile-induced elevation of TNFR1 by 75% at 30 min, TNFR2 and LTBR by almost 100% at 2 h and 1 week respectively ($P < 0.01$); it also lowered NFKB1 expression by 68% at 30 min, but NFKB2 by only ~15% at 1 week (Fig. 4B & C). Knockdown of TNF did not show an effect on either NFKB1 or NFKB2. This indicated that LTA was mostly responsible for the early activation of canonical pathway and less for the later activation of the non-canonical pathway. There must be other molecules playing bigger roles in NFKB2 activation.

As we examined our data further, we noticed that, despite the fact that LTA kept its elevation through the week of experiment, its expression level was on the track of declining as the treatment extended; at the same time, CD40, another powerful activator of non-canonical pathway, was activated along with TNFR2 at 2 h of acid/bile treatment and went up even a lot more towards the end of the experiment. We wondered whether CD40 was the main activator of non-canonical pathway. So we transfected both the original Het1A and LTA-deficient Het1A cells (Het1A^{LTA-}) with a shRNA vector to knock down CD40 (Fig. 4A). Loss of CD40 alone did not affect NFKB1 much but lowered NFKB2 by 75% ($P < 0.01$). In the LTA/CD40 double-negative cells (Het1A$^{LTA-/CD40-}$), NFKB2 almost failed to respond to acid/bile treatment, indicating that CD40 was the main factor for the activation of non-canonical pathway during chronic acid/bile reflux (Fig. 4B & C). In addition, knockdown of CD40 also attenuated bile-activated expressions of intestinal markers including MUC2, KRT20, VIL1 and CDX2 (Fig. 4D), signifying its role in esophageal metaplasia.

3.6. *Overexpression of CCN1 in esophageal epithelial cells suppresses CD40 activation*

Next, we used Het1A^{CCN1+} cells to do the chronic acid/bile experiment. This time, we focused on the core genes involved in non-canonical NFκB signaling. As shown in Table 3, overexpression of CCN1 significantly lowered LTBR, CD40, CD40LG, TRAF2, and CHUK ($P < 0.01$). These results were also supported by immunofluorescence staining (Fig. 5A). In addition, overexpression of CCN1 inhibited CDX2 (Fig. 5B) and NFKB2 (Fig. 5C) activated by chronic acid/bile insults. Taken together, our experiments demonstrated that overexpression of CCN1 attenuates esophageal metaplasia primarily through suppressing CD40-NFKB2-CDX2 pathway.

4. Discussions

The canonical and non-canonical NFκB activation pathways do not exclude each other, and in fact, they have cross-talks at multiple levels

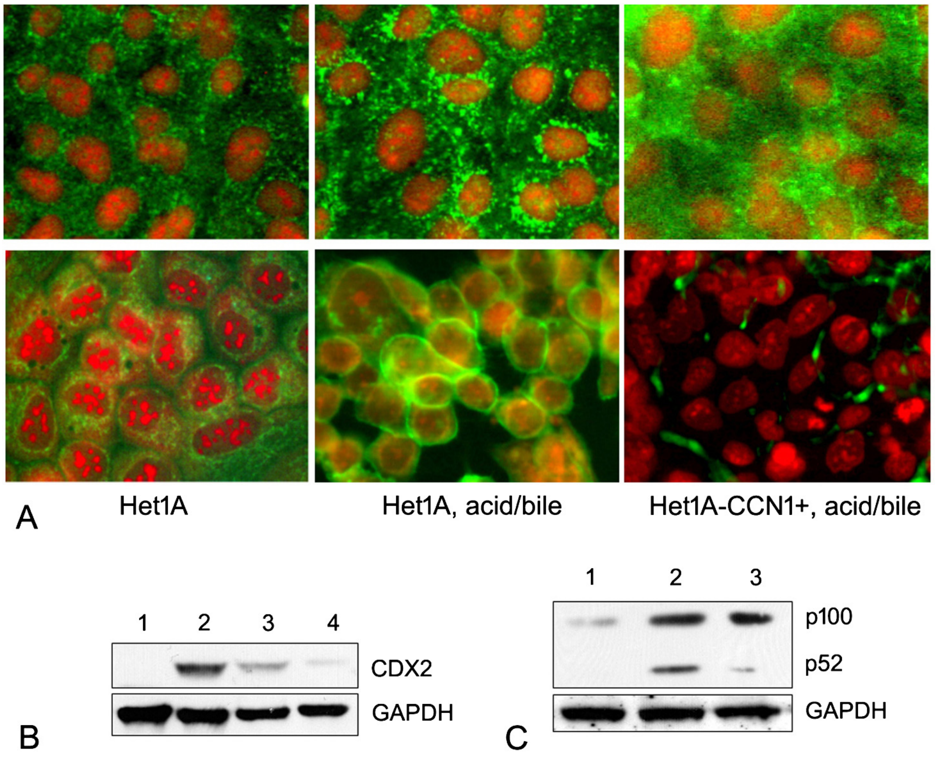

Fig. 5. Overexpression of CCN1 attenuates acid/bile-induced activation of CD40-NFKB2-CDX2 pathway. A. Immunofluorescence staining showing the effect of CCN1 overexpression on CD40 and LTBR. Het1A and Het1A^{CCN1+} cells were subject to chronic acid/bile treatment for a week. The cells were stained for CD40 and LTBR respectively with specific FITC-conjugation antibodies and nuclei were counter-stained with propidium iodide. B. Western blot analysis of CDX2 expression in response to CCN1 overexpression. Lanes: 1. Control, 2. Het1A cells in 1-week chronic acid/bile treatment, 3. Het1A^{CCN1+} cells in 1-week chronic acid/bile treatment, 4. Het1A cells in 1-week chronic acid/bile treatment in the presence of NFκB inhibitor Bay11-7085 at 5 μM. C. Western blot analysis of NFKB2 expression in response to CCN1 overexpression. Lanes: 1. Control Het1A, 2. Control Het1A cells in 1-week chronic acid/bile treatment, 3. Het1A^{CCN1+} cells in 1-week chronic acid/bile treatment.

frequently [29]. At first, NFKB2 is a transcriptional target of NFKB1, and therefore, activation of canonical pathway means to trigger NFKB2 expression; and when NFKB2 is accumulated, it shuts down NFKB1 signaling, vice versa. For this reason, NFKB1 is usually activated immediately after receiving a stimulus, while NFKB2 needs to take a little while to warm up. Once it is activated, however, NFKB2 stays effective much longer than its "twin brother". Secondly, both canonical and non-canonical pathways share a common connector, TRAF2, which can go either way to initiate IKK activation [30]. IKK exists in the cytoplasm as a complex of three proteins: IKKα, IKKβ, and IKKγ (NEMO). The receptors for canonical activation do not have TRAF binding motif and therefore, they rely on TRADD to serve as an adaptor to reach out to TRAF2. However, TRADD does more than just bringing TRAF2 in, it also recruits RIP1 or FADD. RIP1 binds NEMO to activate IKKβ and thereby to supports cell survival, while FADD binds pro-caspase 8 to initiate apoptosis and thereby to put the cell to death. On the contrary, the receptors for activating non-canonical pathway can directly bind TRAF2 and stabilize NIK, and thereby induce IKKα-mediated NFKB2 activation, solely aiming to promote cell survival [31].

Our body is a self-orchestrated biological system, and so is a cell. Every action is associated with a reaction to keep the system in balance. NFκB activation is such an example. NFκB is activated usually due to inflammation or oncogenesis and accordingly, it is often coupled with a negative feedback loop, meaning some NFκB inhibitors get activated as soon as NFκB pathway is activated [32]. For instance, A20 and IκBα are two powerful inhibitors of NFκB signaling and ironically, they both are also the transcriptional targets of NFKB1 [33]. After NFκB activation, whether it stays activated or repressed largely depends on the strength and the duration of the initiating signal. For this reason, the magnitude of NFκB activation is often found in proportion to the number of membrane receptors activated, and the expression level of the responsible receptor often reflects how long the activation would last. Most of the time, the membrane receptor for activating NFκB is downregulated almost immediately after ligand withdrawal. In this study, we can see all of these events taking place during the course of acid/bile treatment. A20 was activated from the very beginning and stayed up all through the experiment while the different pairs of ligand-receptor interactions were taking shifts to meet the instant needs of the inflammatory process.

CD40 was first identified as a marker for B cells and therefore, the majority research about CD40 up to date was done in the context of B cell function, although its expression has been recorded in several other types of cells, including epithelial cells, endothelial cells, fibroblasts as well as other hematopoietic cells [34]. Only a small fraction of CD40 studies has used cells of epithelial origin, and even for those, CD40 expression was exogenously manipulated [35]. Its physiological role in normal epithelial cells is barely known. Here we showed for the first time a huge upregulation of CD40 in normal esophageal epithelial cells in response to chronic acid/bile attacks (simulating GERD episodes), and identified it as a major factor for activating NFκB and the development of intestinal metaplasia. We believe this finding is of great values in drug development for clinical treatment of GERD, BE and even EAC.

GERD is periodic refluxes of a mixture of stomach acid and duodenal bile salts. Based on animal studies, gastric reflux alone does not cause BE or EAC at all. On the contrary, the regurgitated acid might even play a protective role against bile-induced esophageal malignancy. For example, a study demonstrated that 87% of rats with surgically-created duodenal reflux alone developed EAC, while only 30% the rats with gastric-duodenal reflux had such condition, and no rat with gastric reflux alone showed a sign of cancer [28]. These *in vivo* results coincidentally match our *in vitro* study, telling us that acid in GERD episodes might just play a role for triggering inflammatory responses, which is a constructive effort. It is the bile that mediates cellular transformation, which leads to cancer.

Although CCN1 has been known as a double-edged sword, killing some cancer cells while nourishing others [36–38], it has been found in our studies to be a positive factor in the context of gastrointestinal abnormalities, supporting wound healing and normal cell growth [1–3,17] while inducing apoptosis in tumor cells [17,18]. During the course of EAC development from GERD, we found a robust expression of CCN1 in GERD and then it became weaker as the condition moves towards BE and EAC [17]. In this current study, we demonstrated that CCN1 attenuated bile-induced esophageal metaplasia. Taken together, we think that the role of CCN1 in esophageal malignancy is perhaps like this: it gets elevated in esophageal epithelial cells in response to acid exposure to heal the wounded tissue; when the inflammatory process gets out of control and becomes chronic eventually, CCN1 is forced to hold back so that esophageal epithelial cells get a chance to transform into intestinal epithelial phenotype and further become cancerous. Based on this rationale, promotion of CCN1 in GERD patients would help to prevent BE or malignancy.

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Conflict of interest

None.

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