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Cholangiocarcinoma cell proliferation is enhanced in primary sclerosing cholangitis: A role for IL-17A

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Abstract

Primary sclerosing cholangitis (PSC) is a chronic inflammatory disease of the biliary tree and a risk factor for development of cholangiocarcinoma (CCA). The pathogenesis of PSC-related CCA is largely unclear, although it is assumed that chronic inflammatory environment plays a pivotal role. We aimed to investigate the effect of inflammation-related cytokines in PSC on the proliferation rate of cancer cells. For this, the proliferation index in PSC-CCA and sporadic CCA was determined by Ki-67 immunohistochemistry. The percentage of Ki-67 positivity in cancer cells was significantly higher in PSC-CCA than in sporadic CCA (41.3% ± 5.7% vs 25.8% ± 4.1%; $P = .038$). To assess which cytokines in the inflammatory environment have the potential to stimulate cancer cell proliferation, patient-derived CCA organoids (CCAOs) were exposed to five cytokines related to PSC (Interleukin (IL)-1β, IL-6, IL-17A, interferon gamma and tumor necrosis factor alpha). Only IL-17A showed a significant stimulatory effect on cell proliferation in CCAOs, increasing organoid size by 45.9% ± 16.4% ($P < .01$) and proliferation rate by 38% ± 16% ($P < .05$). IL-17A immunohistochemistry demonstrated that PSC-CCA might express more IL-17A than sporadic CCA. Moreover, correlation analysis in sporadic CCA and PSC-CCA found a significant correlation between IL-17A expression and proliferation. In conclusion, tumor cell proliferation is increased in PSC-CCA cells compared with sporadic CCA cells. IL-17A increases CCA cell proliferation in vitro and may contribute to the high proliferation rate in PSC-CCA in situ. Therefore, IL-17A represents a new potential therapeutic target in (PSC-)CCA, to be tested in future trials.

KEYWORDS
cholangiocarcinoma, IL-17, organoid, primary sclerosing cholangitis, proliferation

Abbreviations: CCA, cholangiocarcinoma; CCAO, cholangiocarcinoma organoid; FFPE, formalin fixed paraffin embedded; H&E, hematoxylin & eosin; IFNγ, interferon gamma; IL, interleukin; PSC, primary sclerosing cholangitis; TNFα, tumor necrosis factor alpha.

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What's new?

Primary sclerosing cholangitis (PSC) is a chronic fibrotic inflammatory disease of the biliary tree and a risk factor to the development of cholangiocarcinoma (CCA). Differences between PSC-related and sporadic CCA are poorly studied. Here, we demonstrate that PSC-related CCA has a higher proliferative index compared with sporadic CCA. Moreover, we show that inflammatory cytokine IL-17A is locally expressed in tumor tissue and has a direct proliferation inducing effect on biliary cancer cells cultured as organoids.

1 | INTRODUCTION

Primary sclerosing cholangitis (PSC) is a rare chronic disease of the biliary tree. It is characterized by progressive fibrous strictures of the bile ducts in an inflammatory environment.[1] PSC patients have a lifetime risk of 9–20% to develop cholangiocarcinoma (CCA), a severe type of cancer in the biliary tract.[2] CCA is the most common type of malignancy in PSC patients and the leading cause of death, accounting for 24–58% of deaths.[3,4] Once CCA has developed, curative treatment is limited to surgical removal which is only feasible in a minority of patients due to late diagnosis. Five-year survival of PSC-related CCA (PSC-CCA) is only 10%.[5,6] Compared with sporadic CCA, PSC-CCA occurs at a significantly younger age of 48 years old vs 65 in sporadic CCA.[5]

Biological differences between PSC-CCA and sporadic CCA and related consequences for cancer management have only recently gained attention. The developmental process of PSC-CCA is poorly understood and is presumably a combination of genetic aberrations and a dysregulated immune system, causing a chronic inflammatory environment.[5] Genomic characterization of PSC-CCA demonstrated a heterogeneous mutational landscape containing similar mutations to sporadic CCA, with mutations in *TP53*, *KRAS*, *CDKN2A* and *SMAD4* being the most frequent. However, in contrast with sporadic CCA, the PSC-CCA mutational landscape is independent of their anatomical location along the biliary tree.[7] Of note, the key difference between PSC-CCA and sporadic CCA is the chronic inflammatory environment. PSC patients have massive biliary immune cell infiltration, characterized by a high number of neutrophils and T cells, of which the biliary resident $CD103^+CD69^+CD8^+$ effector memory T cells are the most abundant.[8] These immune cells produce pro-inflammatory and pro-fibrogenic cytokines that sustain inflammation and cause fibrous strictures of the bile ducts.[8,9] For instance, it is known that pro-inflammatory cytokine IL-17A is increased in the periductal area of PSC livers.[10] This cytokine contributed to hepatobiliary injury progression in a microbial PSC mouse model and inhibition of IL-17A producing T helper 17 (T_H17) cells efficiently ameliorated the induced damage.[11] IL-17A also induced an immune-reactive phenotype in bile-derived organoids from PSC patients.[12] IL-17A, together with other cytokines and immune cells, creates a chronic inflammatory environment which has been shown to be a fertile ground for cancer development and is able to promote cell proliferation in several types of cancer.[13] Therefore, we wondered whether PSC-related inflammation has a stimulating effect on the progression of CCA, particularly on the proliferation of CCA cells.

In this study, we aim to investigate the proliferation rate in PSC-CCA in comparison with sporadic CCA by Ki-67 immunohistochemistry, and to analyze the effect of PSC-related pro-inflammatory cytokines on the proliferation of patient-derived CCA organoids (CCAOs).

2 | MATERIALS AND METHODS

2.1 | Tissue samples

Formalin-fixed paraffin-embedded (FFPE) tissue blocks of hepatobiliary resection specimens of PSC-CCA patients (n = 19) were collected as a part of standard clinical care. These specimens were obtained in the Erasmus MC, University Medical Center Rotterdam, the Netherlands, between 1996 and 2019. The PSC-CCA samples were matched to sporadic CCA samples (n = 19) for year of resection, tumor location (distal, perihilar or intrahepatic) and tumor differentiation grade, if possible. Patient characteristics are reported in Supplementary Table S1. 4 μm sections were cut and stained with hematoxylin & eosin (H&E) according to standard procedures. These sections were reviewed by an expert hepatobiliary pathologist, who indicated the tumor region with the highest tumor cell percentage.

2.2 | Ki-67 scoring

A second 4 μm section of the selected FFPE tissue blocks was used for Ki-67 immunohistochemistry. The sections were incubated with a monoclonal antibody for Ki-67 according to standard protocols (Supplementary Materials and Methods). The specificity of the reaction was confirmed by positive controls, which was tonsil tissue. Expression of Ki-67 was scored according to a consensus of two investigators (Eline J. C. A. Kamp and Michail Doukas), which were both blinded for all samples. The scoring systems are described in Supplementary Materials and Methods and are based on the analytical validation of Ki-76 scoring in breast cancer.[14]

2.3 | Cytokine receptor gene expression analysis

Gene expression data of CCA tissue (n = 36) was obtained from The Cancer Genome Atlas Research Network (https://www.cancer.gov/tcga). In parallel, gene expression data of CCAO lines included in this

study (n = 3) was obtained from the Gene Expression Omnibus (GEO) repository with accession number GSE84073 (CCAO1) and GEO repository with accession number GSE179601 (CCAO2 and CCAO3). Normalized counts were obtained from all datasets for receptors of PSC-related cytokines interleukin (IL)-1β, IL-2, IL-6, IL-17A, interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα).

2.4 | Organoid culture

CCAOs were initiated from patient tumor samples (n = 4) collected at Erasmus MC Rotterdam. Collection and use of patient material in this study was approved by the medical ethics committee of the Erasmus MC (MEC-2013-143). Patients consented to donate resected materials for research purposes. Organoids were cultured as described previously.[15] Confirmation that the organoids were composed of neoplastic cells was obtained by tumor formation after subcutaneous xenografting in female NOD.Cg-Prkdc^{SCID} Il2rg^{tm1Wjl}/SzJ (NSG) mice (Charles River) and by detection of DNA aberrations by targeted next generation sequencing.

2.5 | Targeted next generation sequencing

Next generation sequencing (NGS) was performed in all CCAOs (n = 4) to confirm their tumor cell composition. DNA of organoid cultures was isolated according to standard procedures with 5% Chelex 100 resin and proteinase K. Both mutations and copy number variants were analyzed by using a tailor-made gene panel. Details are further described in the Supplementary Materials and Methods and in Supplementary Table S2. The sequencing coverage and quality statistics for each sample are summarized in Supplementary Table S3.

2.6 | Cytokine exposure

On day 0, organoids were split 1:3–1:12 and replated in fresh basement membrane extract (Cultrex). They were covered in organoid expansion medium supplemented with 2, 10, 50 or 200 ng/ml of cytokines IL-1β (Bio-Techne), IL-6 (Life Technologies Europe BV), IL-17A (Merck Life Science NV), IFNγ (Life Technologies Europe BV) and TNFα (Peprotech). A vehicle control (IL-1β, IL-6, IFNγ: PBS with 0.1% BSA; IL-17A: distilled water; TNFα: DMSO; diluted 500 times in culture medium) was included in every experiment. Medium with fresh cytokines was added at day 3. Readouts were performed at day 5.

2.7 | Quantification of proliferation

Organoid proliferation was quantified using the Click-it EdU flow cytometry assay kit (Invitrogen) according to manufacturer's guidelines. Organoid cultures were incubated with 10 μM EdU for 4 h. Consecutively, organoids were dissociated to single cells using Trypsin-EDTA (15-60 min at 37 °C), fixed, permeabilised and stained with Alexa Fluor 488. Flow cytometry was performed with FACSCanto II flow cytometer (BD Biosciences) and analyzed using FlowJo software (version 10.7, LLC). Gating strategy is displayed in Supplementary Figure S1.

2.8 | Bright field and Live/dead immunofluorescent imaging

Bright field and fluorescence imaging was performed with an EVOS FL Cell Imaging System equipped with RFP, GFP and DAPI light cubes. Live/dead staining mix consisted of 12.5 μg/ml Propidium Iodide (PI, Sigma-Aldrich), 10 μg/ml Hoechst 33342 (Thermo Fisher Scientific) and 0.5 μM Calcein AM (Thermo Fisher Scientific). Cytokine treated organoids were stained for 1 h before imaging. Organoid diameter measurement is described in Supplementary Materials and Methods.

2.9 | IL-17A immunohistochemistry and scoring

A third section of the selected FFPE tissue blocks was used for IL-17A immunohistochemistry. The sections were incubated with monoclonal antibodies of IL-17A according to standard protocols. The IL-17A scoring was performed by an expert hepatobiliary pathologist (MD) and was based on his experience and previously published literature.[16] Details of the staining and scoring method are described in the Supplementary Materials and Methods.

2.10 | Statistical analysis

Statistical analyses were performed using GraphPad Prism software (v. 8.0.2). Values are portrayed as mean ± SEM. Comparisons between two matched groups were analyzed by a two-tailed paired t test or a Wilcoxon test. Comparisons between two unmatched groups were analyzed by a two-tailed unpaired *t* test or a Mann-Whitney test. *P* values below .05 were deemed statistically significant.

3 | RESULTS AND DISCUSSION

3.1 | Increased proliferation in PSC-CCA compared with sporadic CCA

A total of 19 PSC patients with CCA were included, and matched to 19 patients with sporadic CCA (Supplementary Table S1). Each group of patients contained 11 perihilar, four intrahepatic and four distal CCA. The age of CCA diagnosis was significantly lower in PSC-CCA compared with sporadic CCA (*P* = .000). There was no statistically significant difference between the two used scoring systems for Ki-67 positivity, global and hotspot, in both PSC-CCA and sporadic CCA. The mean of pathological estimation and these scoring systems resulted in a significantly higher percentage of Ki-67 positive tumor cells

in PSC-CCA when compared with sporadic CCA (41.3% ± 5.7% vs 25.8% ± 4.1%; mean ± SEM, $P = .038$) (Figure 1A, B), indicating PSC-CCA tumor cells proliferate faster than their sporadic CCA counterparts.

Few other studies have performed Ki-67 immunohistochemistry in PSC-CCA. Obviously, Ki-67 positivity was significantly higher in CCA than in non-neoplastic bile ducts.[17,18] Ishii et al have analyzed Ki-67 in both PSC-CCA and sporadic CCA before, and found a similar trend with 46% Ki-67 positivity in PSC-CCA compared with 33% in sporadic CCA.[19] However, their analysis did not reach significance, most likely due to the low sample size (7 PSC-CCAs vs 15 sporadic CCAs).

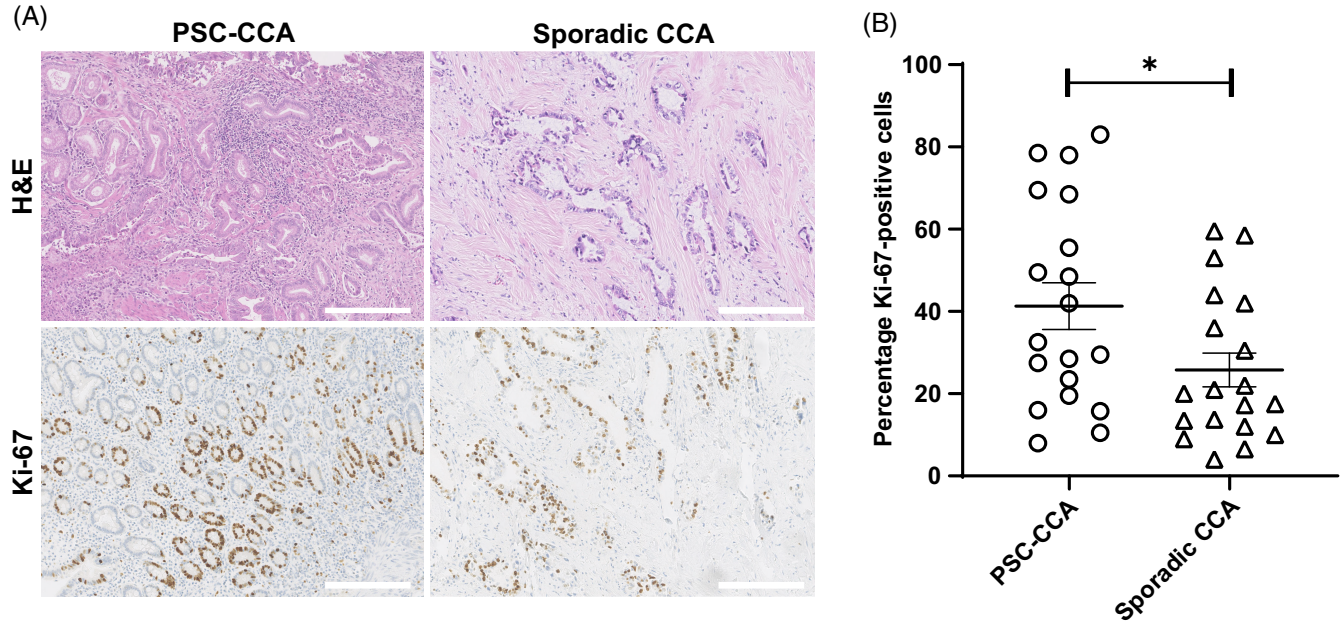

FIGURE 1 Proliferation rate in PSC-CCA is higher than sporadic CCA. Ki-67 immunohistochemistry in 19 PSC-CCA samples and 19 sporadic CCA samples. (A) Representative images of Ki-67 staining in a matched sample of PSC-CCA (left) and sporadic CCA (right). Original magnification, ×10. Scale bar = 250 μm. (B) Quantification of Ki-67 staining in tumor cells as determined by two independent observers (*$P < .05$). All values with error bars display mean ± SEM. [Color figure can be viewed at wileyonlinelibrary.com]

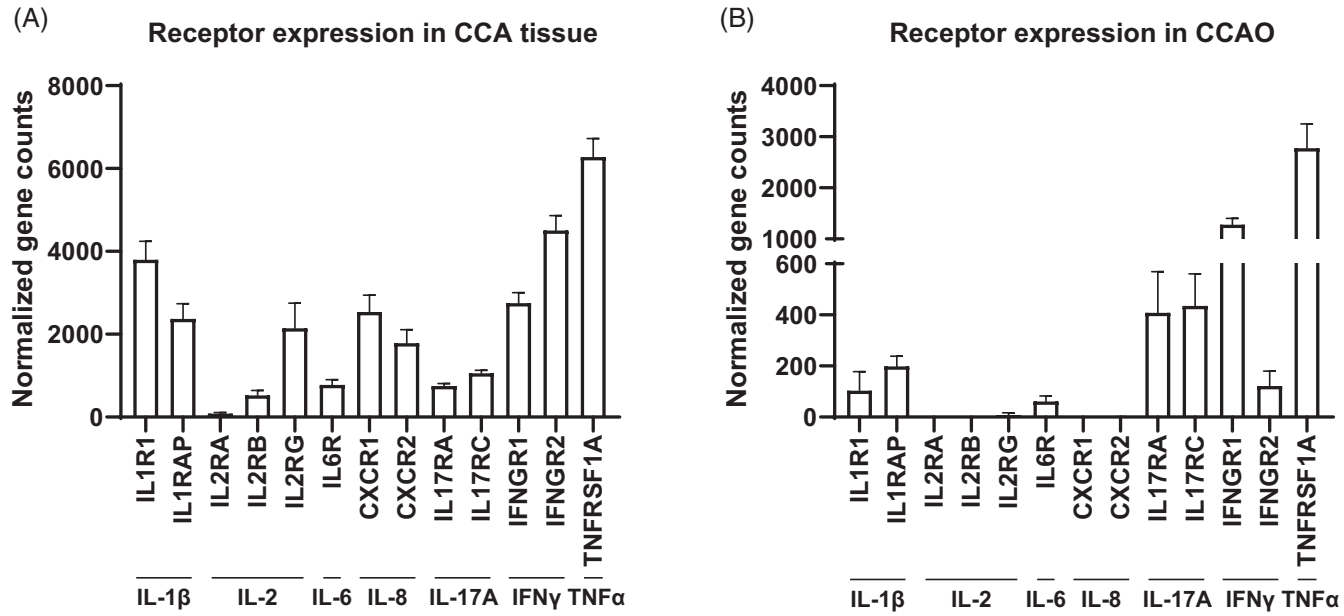

FIGURE 2 Gene expression of cytokine receptors in CCA tissue and CCAOs. PSC- and PSC-CCA related cytokines were selected from literature. (A) Gene expression of selected cytokine receptors in CCA tissue sections (n = 36) as obtained from The Cancer Genome Atlas Research Network. (B) Gene expression of selected cytokine receptors in CCAOs (n = 3). All values with error bars display mean ± SEM.

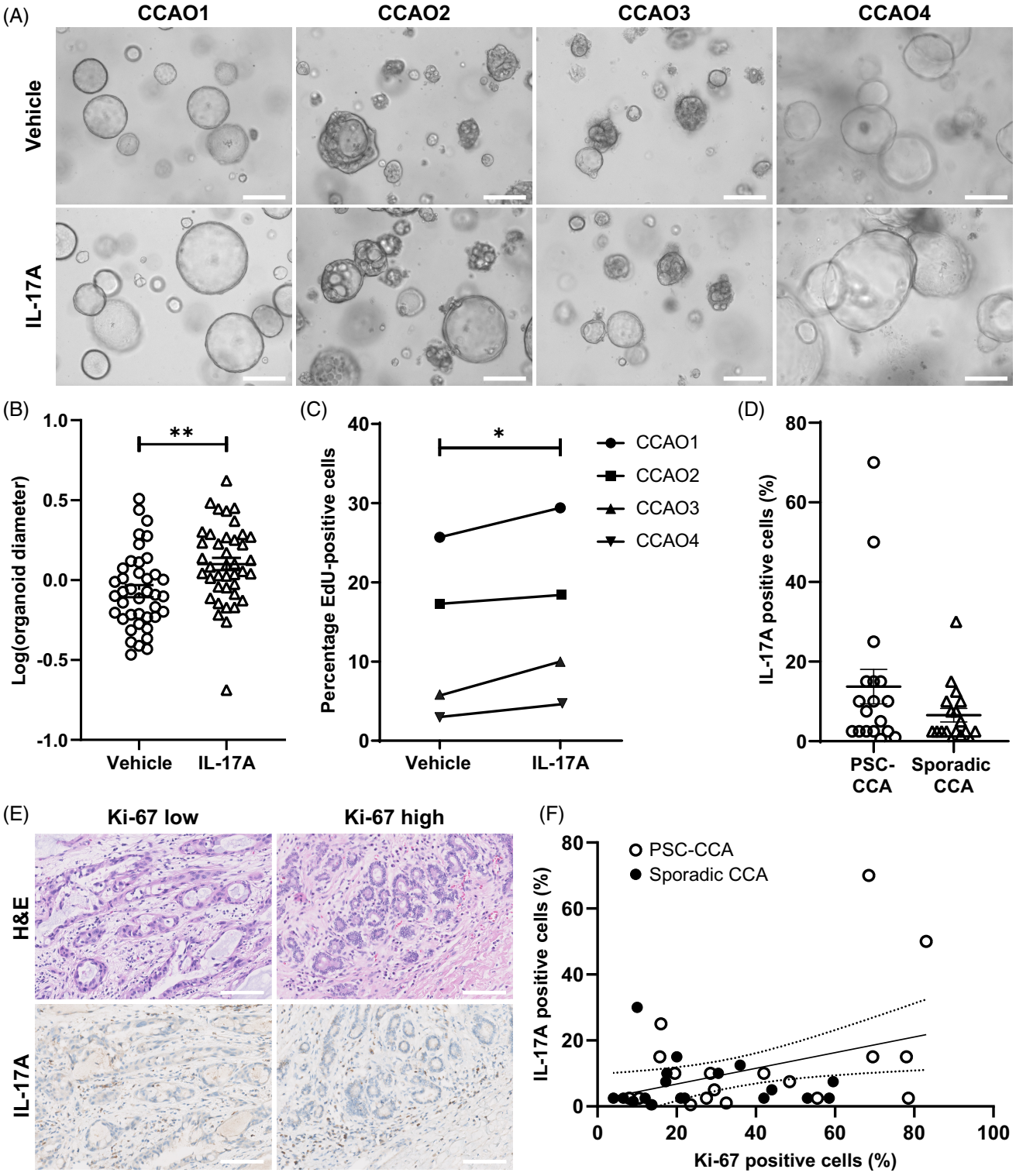

FIGURE 3 IL-17A stimulates proliferation of CCAOs. CCAOs were cultured for 5 days with 200 ng/ml IL-17A or with vehicle added to the medium. (A) Bright field images demonstrate morphology is not affected by 200 ng/ml IL-17A treatment. Scalebar = 200 μm. (B) Log transformed organoid diameters as measured from bright field images relative to the mean of vehicle treated organoids (n = 10 organoids per CCAO line per condition; n = 4 organoid lines). Organoid diameter is significantly larger when treated with 200 ng/ml IL-17A (**P < .01). (C) Percentage of EdU-positive cells in each CCAO line treated with 200 ng/ml IL-17A. The percentage of EdU-positive cells is significantly higher in CCAOs treated with IL-17A compared with the vehicle treated ones (*P < .05). (D) Quantification of IL-17A staining in 18 PSC-CCA and 18 sporadic CCA samples as determined by an experienced GI-pathologist. (E) Hematoxylin and Eosin (H&E) staining and IL-17A immunohistochemistry performed in PSC-CCA with a low Ki-67 positivity score (left) and PSC-CCA with a high Ki-67 positivity score (right). The IL-17A score was determined by the percentage of all positive cells. Original magnification, ×20. Scalebar = 100 μm. (F) Correlation plot of the number of IL-17A positive cells and the Ki-67 positive cells in PSC-CCA and sporadic CCA samples (n = 18 each). All values with error bars display mean ± SEM. [Color figure can be viewed at wileyonlinelibrary.com]

Despite the fact that our study identified an increased proliferation in PSC-CCA compared with sporadic CCA, a multi-center study recently found no difference in survival after resection between perihilar PSC-CCA and sporadic CCA.[20] Various factors could be involved, including the younger age of PSC-CCA patients, which means they are probably physically more fit with less comorbidity than older patients. The median age of diagnosis of CCA in PSC patients is about 20 years earlier than patients with CCA without PSC.[2] The younger PSC-CCA patients are more likely to undergo additional treatments than older CCA patients, like chemotherapy. In addition, the intensive follow-up of PSC patients might result in an earlier detection of CCA.

3.2 | Testing cell proliferation in a CCA organoid (CCAO) model

As the number of proliferating cancer cells is higher in PSC-CCA compared with sporadic CCA, we aimed to identify the direct effect of PSC-related inflammatory cytokines on CCA cell proliferation. In order to investigate this, we established CCAOs that provide an epithelial cancer cell model and confirmed their tumorigenicity by xenografting and targeted NGS. The cancer diagnostic NGS panel described in Supplementary Table S2 revealed CCAO copy number variations and mutations in *ARID1A*, *IGF1R*, *KRAS*, *TP53* and *BAP1* (Supplementary Figure S2A, B). *TP53*, *KRAS* and *ARID1A* mutations were also regularly identified in PSC-CCA.[7]

Next, we performed a literature search into PSC-related inflammatory cytokines that identified IL-1β, IL-2, IL-6, IL-8, IL-17A, IFNγ and TNFα.[8,21,22] These cytokines could only exert their effects on CCA cells if their receptors are present. Therefore, we analyzed cytokine receptor gene expression in CCA tumor tissue and CCAOs. CCA transcriptomic data extracted from The Cancer Genome Atlas ($n = 36$) shows that receptors of all cytokines are expressed in CCA bulk tumor samples, except for IL2RA (Figure 2A). In CCAOs, which only consist of epithelial tumor cells, receptors are expressed for IL-1β, IL-6, IL-17A, IFNγ and TNFα (Figure 2B). Therefore, these five cytokines were selected to analyze in organoid exposure experiments.

3.3 | IL-17A stimulates CCA cell proliferation

To assess which of the selected cytokines are able to stimulate proliferation, the cytokines were added to CCAOs. CCAOs were exposed to IL-1β, IL-6, IL-17A, IFNγ and TNFα for 5 days. Of these, IL-1β, IL-6, IFNγ and TNFα did not increase the CCAO proliferation rate (data not shown). However, incubation with IL-17A showed an increase in organoid diameter. CCAOs treated with IL-17A were 45.9% ± 16.4% (mean ± SEM, $P < .01$) bigger compared with vehicle treated organoids (Figure 3B). Also, IL-17A treatment significantly increased the CCAO cell proliferation by 38% ± 16% (mean ± SEM) compared with the proliferation in vehicle treated CCAOs (Figure 3C). IL-17A had no further impact on organoid morphology (Figure 3A), nor did it induce cell death (Supplementary Figure S3). These findings indicate that IL-17A is able to boost CCA cell proliferation directly. The direct proliferation inducing effect of IL-17A has never been studied in CCA, but it has been described in colorectal cancer models. IL-17A increased the growth rate of premalignant enterocytes in colorectal cancer mouse models and murine organoids, which coincided with increased activity of ERK, p38 MAPK and NF-κB signaling.[23] Furthermore, in colon cancer cell line CT26, IL-17A overexpression enhanced cell cycle progression.[24] Clinically, intra- and peritumoral IL-17A tissue expression are poor prognostic factors in sporadic CCA patients.[25-27] Faster cancer cell proliferation induced by IL-17A could potentially play a role in the poor survival of these patients.

3.4 | IL-17A is expressed in PSC-CCA tissue

Next, we examined if proliferation and IL-17A expression were related in PSC-CCA patients. In PSC without malignancy, it is known that IL-17A and its producers T helper 17 (Th17) cells are overrepresented and overactive. Blood of PSC patients contains more Th17 cells compared with healthy individuals,[28] and has a stronger Th17 response upon pathogen stimulation.[10] Single cell RNA sequencing of the intrahepatic T cells in PSC livers identified a naïve-like $CD4^+$ T cell population with a predisposition to Th17 cell polarization.[29] Furthermore, immunohistochemistry detected a higher number of IL-17A positive cells in the periductal area of PSC patients.[10] However, little is known about the expression of IL-17A in PSC-CCA. Therefore, IL-17A immunohistochemistry was performed on PSC-CCA and sporadic CCA tumor tissue samples (Figure 3D). The percentage of IL-17A positive cells was higher in PSC-CCA (13.7% ± 4.3%) compared with sporadic CCA (6.5% ± 1.7%), although this did not reach statistical significance ($P = .08$). To further investigate the specific relationship between proliferation and IL-17A, correlation analysis was performed between the number of IL-17A positive cells and the number of Ki-67 positive cells in both PSC-CCA and sporadic CCA (Figure 3E, F). Linear regression demonstrated a significant correlation ($P = .018$), although the correlation coefficient was relatively low (R square $= 0.14$), likely due to a high degree of variation. Combined with the demonstrated proliferation-inducing effect of IL-17A on CCA cancer cells, these findings suggest that IL-17A might be higher expressed in PSC-CCA compared with sporadic CCA and could be a contributing factor to the high proliferation of PSC-CCA tumors.

4 | CONCLUSION

In this study, we investigated the effect that a PSC background has on CCA cell proliferation and studied the role of inflammatory cytokines therein. We found that the proliferative index of cancer cells in PSC-CCA is higher than that found in sporadic CCA. Moreover, we found that cytokine IL-17A is locally expressed in tumor tissue and stimulates cell proliferation in vitro in CCAOs. This indicates IL-17A might be a contributing factor to the higher proliferative index in PSC-CCA. Future studies could focus on the potential of IL-17A

targeted therapies in (PSC-)CCA treatment, especially in patients with highly proliferative tumors.

AUTHOR CONTRIBUTIONS

Ruby Lieshout, Eline J. C. A. Kamp, Monique M. A. Verstegen, Ruby Lieshout, Eline J. C. A. Kamp, Monique M. A. Verstegen and Annemarie C. de Vries conceived the idea and designed the study. Ruby Lieshout and Eline J. C. A. Kamp performed the experiments and analyzed the data. Kübra Köten assisted with experiments. Michail Doukas and Eline J. C. A. Kamp evaluated immunohistochemistry slides. Ruby Lieshout, Eline J. C. A. Kamp, Monique M. A. Verstegen, Luc J. W. van der Laan and Annemarie C. de Vries contributed to the interpretation of the results. Ruby Lieshout and Eline J. C. A. Kamp wrote the manuscript. All authors critically revised the manuscript. The work reported in the paper has been performed by the authors, unless clearly specified in the text.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The study was approved by the local Medical Ethics Review Board (MEC-2013-143).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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