The role of duodenal innate lymphoid cells in familial adenomatous polyposis

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List of abbreviations

aFAP	Attenuated familial adenomatous polyposis
Ahr	Aryl hydrocarbon receptor
AMPs	Antimicrobial proteins
ANOVA	Analysis of variance
APC	Adenomatous polyposis coli
APC$^{min/+}$ mice	FAP mouse model with APC mutation bearing multiple intestinal neoplasia (min)
AREG	Amphiregulin
BFA	Brefeldin A
BSA	Bovine Serum Albumin
CAPN8	Calpain 8
CD	Cluster of differentiation
CDH3	Cadherin 3
cDNA	Complementary DNA
CD103	Integrin, alpha E
CD117	Receptor tyrosine kinase ckit
CD127	Interleukin-7 receptor-α
CEMIP	Cell migration-inducing and hyaluron-binding protein
CODEX	Co-detection by indexing
CO_2	Carbon dioxide
CRC	Colorectal cancer

cRPMI	Complete cell culture medium
CRTH2	Prostaglandin D2 receptor 2
DCs	Dendritic cells
DEG	Differentially expressed gene
DL	Delta-Like
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's Phosphate-buffered saline
DTT	Dithiothreitol
Duox	Dual oxidase
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EOMES	Eomesodermin
FAP	Familial adenomatous polyposis
FBS	Fetal Bovine Serum
FDR	False discovery rate
Fig.	Figure
GATA3	GATA-binding protein 3
GI	Gastrointestinal
GCDR	Gentle Cell Dissociadion Reagent
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H_2O_2	Hydrogen peroxide
HBSS	Hanks Balanced Salt Solution

HCC	Hepatocellular carcinoma
HSA	Human serum albumin
IBD	Inflammatory bowel disease
ie	Intraepithelial
IECs	Intestinal epithelial cells
IFN-γ	Interferon-gamma
IL-	Interleukin-
ILC	Innate lymphoid cell
ILCP	ILC precursor
IL-2Rγc	interleukin-2 receptor gamma common chain
KLR	Killer cell lectin-like receptor
KRT7	Keratin 7
KW	Kruskal-Wallis
LGR5	Leucin-rich repeat-containing G-protein coupled receptor 5
Lin	Lineage
LP	Lamina propria
LS	Lynch syndrome
LTi	Lymphoid tissue inducer
LYZ	Lysozyme
MAPK	Mitogen-activated protein kinase
MELC	Multi-Epitope-Ligand-Cartography
MFI	Mean fluorescent intensity

mRNA	Messenger RNA
muc-2	Mucin-2
NAC	N-acetylcysteine
NAP	Nucleic acid preservation buffer
NF-κB	Nuclear factor κ-light-chain-enhancer of activated B cells
NK	Natural killer
NKp44	Natural cytotoxicity triggering receptor 2
OGM	Organoid Growth Medium
P/S	Penicillin-Streptomycin
PBS	Phosphate-buffered saline
PBMCs	Peripheral blood mononuclear cells
PC	Principal component
P/I	PMA/Ionomycin
PFA	Paraformaldehyde
PMA	Phorbol 12-myristate 13-acetate
qRT-PCR	Quantitative real-time polymerase chain reaction
RAG	Recombination activating gene
RNA	Ribonucleic acid
ROR	Retinoic acid-related orphan receptor
RT	Room temperature
scRNAseq	Single-cell RNA sequencing
SCC	Squamous cell carcinoma

SD	Standard deviation
Seq	Sequencing
SIgA	Secretory immunoglobulin A
SS	Spigelman stage
S100P	S100 Calcium binding protein P
Tab.	Table
T-bet	T-box transcription factor TBX21
TF	Transcription factor
TGF-β1	Transforming growth factor beta 1
Th	T helper cells
TL1A	Tumor necrosis factor-like cytokine 1A
TME	Tumor microenvironment
TNF-α	Tumor necrosis factor alpha
Treg	Regulatory T cell
TSLP	Thymic stromal lymphopoietin
TSPAN1	Tetraspanin 1
WT	Wildtype
-/-	Knock-out

1. Introduction

1.1 Familial Adenomatous Polyposis

Familial adenomatous polyposis (FAP), an autosomal dominant inherited gastrointestinal (GI) tumor syndrome is characterized by the development of multiple (100-1000) adenomatous polyps in the colon and rectum (Gryfe, 2009; Petersen *et al.*, 1991; Nishisho *et al.*, 1991). The prevalence of FAP is reported to be 1:6850 to 1:31250 with no regional differences (Yen *et al.*, 2022). Men and women are affected with equal frequency (Syngal *et al.*, 2015; Yen *et al.*, 2022).

Adenoma formation commonly starts during childhood. The first symptoms appear at an average age of 16 years (Yen *et al.*, 2022; Stec *et al.*, 2010; Yang *et al.*, 2021a). With 35 years, 95 % of FAP patients are diagnosed with polyps (Yen *et al.*, 2022; Yang *et al.*, 2021a). The adenomatous polyps have the potential to develop into cancerous tumors if left untreated (Yen *et al.*, 2022). The development of colorectal cancer (CRC) is almost inevitable in patients with classical FAP and the risk increases with age. In untreated patients, the average age of CRC diagnosis is 39 years (Yen *et al.*, 2022; Yang *et al.*, 2021a; Stec *et al.*, 2010). To reduce the risk of cancer development and improve their quality of life, individuals with FAP are typically advised to undergo regular surveillance and prophylactic colectomy (Lynch and La Chapelle, 1999; Stec *et al.*, 2010; Campos, 2014; Nieuwenhuis and Vasen, 2007; Campos *et al.*, 2015; Yen *et al.*, 2022).

FAP is caused by heterozygous germline mutations of the adenomatous polyposis coli (*APC)* gene, which is a tumor suppressor gene (Aitchison *et al.*, 2020; Testa *et al.*, 2018; Bodmer *et al.*, 1987; Groden *et al.*, 1991). According to Knudson's 2-Hit hypothesis, heterozygous carriers of a germline mutation need a somatic mutation in the second allele to induce adenoma formation by loss of heterozygosity (Knudson, 1971). The *APC* gene is located on chromosome 5q21-q22 and consists of 15 exons encoding a protein of 2843 amino acids with a molecular weight of 309 kilodalton (Barber *et al.*, 1994; Plawski and Slomski, 2008; Plawski *et al.*, 2004; Brosens *et al.*, 2005; Aitchison *et al.*, 2020; Bodmer *et al.*, 1987). Genotype-phenotype correlations in colonic polyposis are well established and show that in FAP mutations at the 5' part of exon 15 are most frequently

mutated within the mutation cluster region between codons 1286 and 1513 (Miyoshi *et al.*, 1992; Aitchison *et al.*, 2020; Newton *et al.*, 2012; Nieuwenhuis *et al.*, 2009). Typical mutations at codon 1061 and 1309 lead to an early development of adenomas and CRC (Fig. 1; Brosens *et al.*, 2005; Stec *et al.*, 2010).

A milder form of FAP named attenuated FAP (aFAP) has mutations mostly at the ends of the *APC* gene (Fig. 1, Brosens *et al.*, 2005). AFAP is associated with fewer colonic (<100) polyps and a later onset of CRC (Brosens *et al.*, 2005).

The majority of *APC* mutations lead to a truncated form of APC resulting in a loss of function (Plawski *et al.*, 2004). In the normal intestinal mucosa, the APC protein is a tumor suppressor of the Wnt signaling pathway (Hankey *et al.*, 2018).
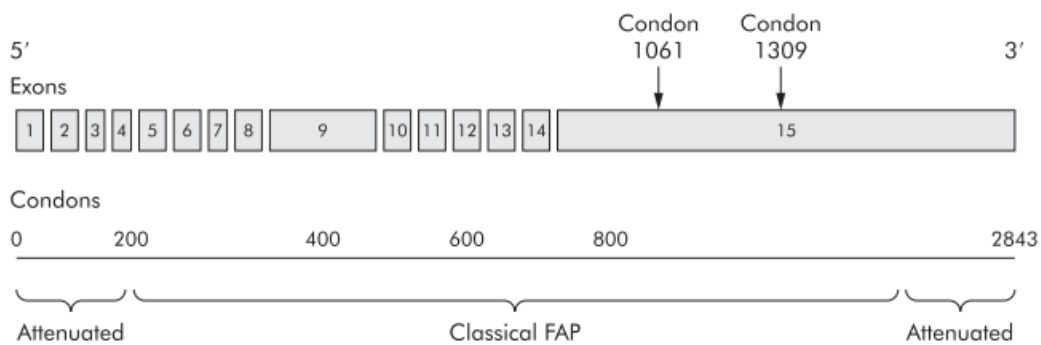
Fig. 1: Schematic representation of the adenomatous polyposis coli (APC) gene. Most mutations in FAP occur in codon 1061 and 1309 whereas aFAP mutations classically appear at the ends of the *APC* gene. (adapted from Brosens *et al.*, 2005)

The Wnt signaling pathway controls the cell cycle, proliferation, migration, differentiation and apoptosis. The APC protein interacts with β-catenin, leading to degradation of β-catenin by the proteasome along with GSK-3β, CK1α and axin (degradation complex) (Logan and Nusse, 2004; Testa *et al.*, 2018). When Wnt binds to the N-terminal extra-cellular cysteine-rich region of a Frizzled family receptor, a member of the superfamily of G-protein-coupled receptors, the degradation complex is inhibited (Pai *et al.*, 2017; Logan and Nusse, 2004). That leads to the accumulation of β-catenin in the cytoplasm, its translocation into the nucleus and after binding T-cell transcription factor or

lymphoid enhancer factor to the transcription of Wnt target genes (Pai *et al.*, 2017; Logan and Nusse, 2004).

The mutated APC protein loses its ability to bind and degrade β-catenin, so that the Wnt/β-catenin pathway is permanently activated (Fig. 2). As a result, β-catenin causes the transcription of its target genes without a Wnt signal (Logan and Nusse, 2004). The dysregulation of the Wnt signaling pathway is associated with the development of tumors in humans (Lecarpentier *et al.*, 2019).
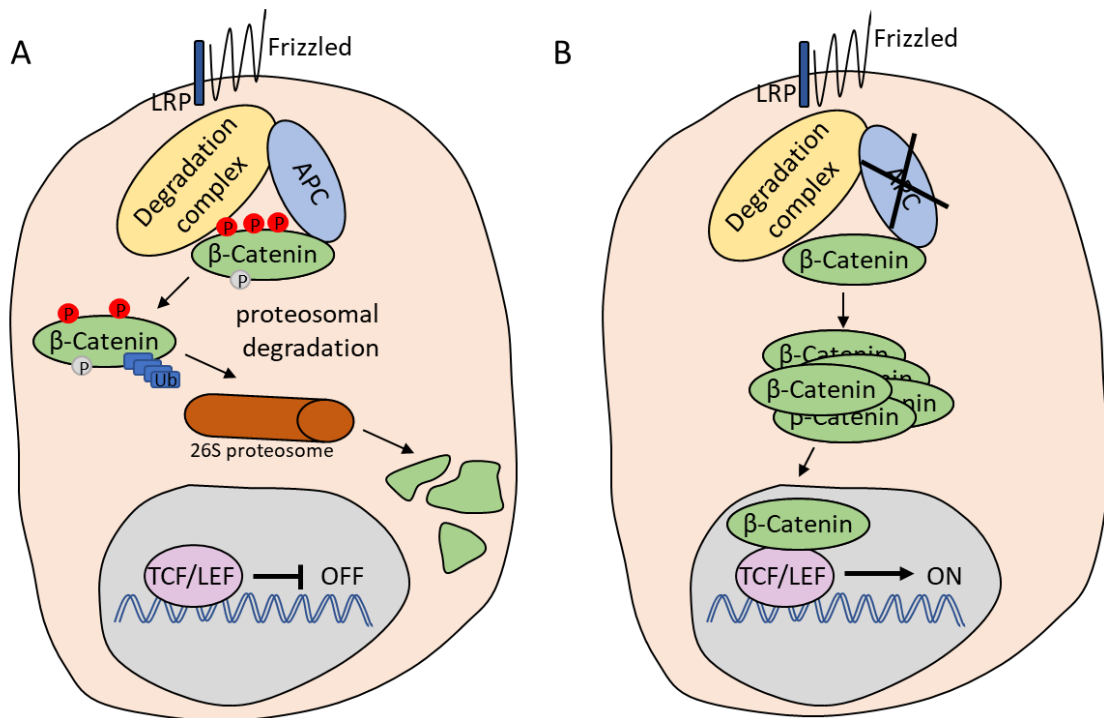
Fig. 2: APC function in the Wnt signaling pathway. (A) Functional APC assists in targeting β-catenin for degradation. (B) Loss of APC leads to accumulation of β-catenin and the transcription of Wnt target genes after binding T-cell transcription factor (TCF) or lymphoid enhancer factor (LEF). (adapted from Rusan and Peifer, 2008; Jeong *et al.*, 2018).

Besides the development of colorectal adenomas and cancer, FAP is also associated with numerous extra-colonic manifestations such as gastric and duodenal adenomas, osteomas, dental abnormalities, congenital hypertrophy of the retinal pigment epithelium, soft tissue tumors, desmoid tumors, and other malignancies such as thyroid carcinomas, malignant central nervous system tumors (medulloblastoma), or hepatoblastomas

(Jasperson *et al.*, 2010; Campos *et al.*, 2015; Yen *et al.*, 2022; Gurbuz *et al.*, 1994; Groen *et al.*, 2008).

The development of duodenal adenomas is the most common extra-colonic manifestation of FAP, with a lifetime risk of developing duodenal adenomas of nearly 100 % (Brosens *et al.*, 2005; Bülow *et al.*, 2004). As a result, FAP patients have a significantly higher risk (4-12 %) of developing duodenal carcinoma than the general population (Brosens *et al.*, 2005; Jasperson *et al.*, 2010; Latchford *et al.*, 2009).

To classify duodenal polyposis, a staging system was developed by Spigelman *et al.* in 1989 that included number, size, histology, and degree of dysplasia of duodenal polyps (Spigelman *et al.*, 1989). Based on a score of the Spigelman staging (SS) system (Tab.1), patients can be classified into five different stages (0-IV), with stage 0 being present in the absence of polyps and stage IV representing severe polyposis (Spigelman *et al.*, 1989). Since there is an association between SS and the risk of developing duodenal carcinoma this classification forms an important basis for determining the intervals between screenings and therapeutic intervention options (Dinarvand *et al.*, 2019).

Tab. 1: Spigelman stages for grading duodenal polyposis in FAP. (adapted from Groves *et al.*, 2002b)

Criteria	Points		
	1	2	3
Polyp number	1-4	5-20	>20
Polyp size (mm)	1-4	5-10	>10
Histology	Tubular	Tubulovillous	Villous
Dysplasia	Mild	Moderate	Severe
Stage 0: 0 points, stage I: 1-4 points, stage II: 5-6 points, stage III: 7-8 points, stage IV: 9-12 points			

Based on the observation that patients with severe polyposis have a higher risk of developing duodenal carcinoma (Bülow *et al.*, 2004), duodenal adenomas are also thought to follow the adenoma-carcinoma sequence in a manner similar to colon adenomas (Spigelman *et al.*, 1994; Muto *et al.*, 1975). In addition, adenoma tissue either as a component of or adjacent to duodenal carcinoma has been demonstrated in over

90 % of malignancies (Spigelman *et al.*, 1994). However, the growth of duodenal adenomas is much slower than that of adenomas in the colon (Burke *et al.*, 1999).

For colorectal manifestations, mutations associated with severe or early disease or mild progression have been described (Nieuwenhuis and Vasen, 2007). The correlation between *APC* mutation and duodenal polyposis is not as clear as previous studies have yielded conflicting results. Whereas Friedl *et al.* failed to identify any clear relationship, Bertario *et al.* observed a distinct pattern of disease for mutations between codon 976 and 1067 and other studies demonstrated a severe phenotype in carriers of mutations in exon 15, particularly distal to codon 1400 (Friedl *et al.*, 2001; Bertario *et al.*, 2003; Groves *et al.*, 2002a).

Consistent with these conflicting results, duodenal phenotype and clinical course are found to vary even between carriers of the same genetic variant, suggesting that factors other than genotype play a role (Brosens *et al.*, 2005; Takao *et al.*, 2021). The local immune system is of particular interest in this context, as many studies have confirmed the impact of local immune responses on development, progression, and treatment outcomes in a variety of different tumors.

1.2 The gastrointestinal immune system

To protect the GI tract from digested exogenous microorganisms and pathogens, there are a variety of intestinal barriers belonging to the immune system (Fig. 3). The surface of the GI tract is covered by a layer of intestinal epithelial cells (IECs) which are organized in a crypt and villus pattern (Montalban-Arques *et al.*, 2018; Peterson and Artis, 2014). The main IECs in the intestine are enterocytes (Fedi *et al.*, 2021). They have a microvilli structure, which gives the intestine the largest epithelial surface area in the human body and allow efficient nutrient absorption (Mason *et al.*, 2008). The IECs are attached to one another and are sealed at their apical borders by tight junctions to prevent uptake of small molecules (Mason *et al.*, 2008). Within the crypts, there are pluripotent intestinal epithelial stem cells which renew the surface continuously and can differentiate into all other intestinal cell types (Peterson and Artis, 2014; Fedi et al., 2021). Paneth cells also reside

in the crypt and secrete antimicrobial proteins (AMPs) to establish and maintain the intestinal microbiota (Lueschow and McElroy, 2020).

The microvilli are covered by a mucus layer produced by goblet cells located in between the enterocytes, which allows nutrients to pass through and prevents pathogens and larger molecules from entering (Mason *et al.*, 2008). The mucus layer also prevents direct contact between epithelial cells and the microbiota (Murphy and Weaver, 2018; Montalban-Arques *et al.*, 2018). Moreover, the mucus layer contains secretory immunoglobulin A (SIgA) which binds to the microbiota and prevents them from attaching to the epithelial cells. The gut microbiome consists of trillions of microorganisms that live in a symbiotic relationship with their host (Murphy and Weaver, 2018; Bevins and Salzmann, 2011). Even if the composition of the microbiota varies between individuals, the commensal microbiota plays an important role in the maintenance of the intestinal barrier functions and regulation of the mucosal immune system (Bevins and Salzmann, 2011). Other IECs are enteroendocrine cells producing hormones that have a key role in food digestion, tissue repair and enterocyte differentiation (Bevins and Salzman, 2011; Fedi *et al.*, 2021; Gribble and Reimann, 2019). The basolateral surface of the IECs associates with the intestinal lamina propria (LP). Not only IECs and the microbiota have functions in maintaining tissue homeostasis and prevent pathogens from entering, also immune cells within the intraepithelial layer and in the LP crosstalk with each other to maintain homeostasis and protect the barrier from external pathogens.
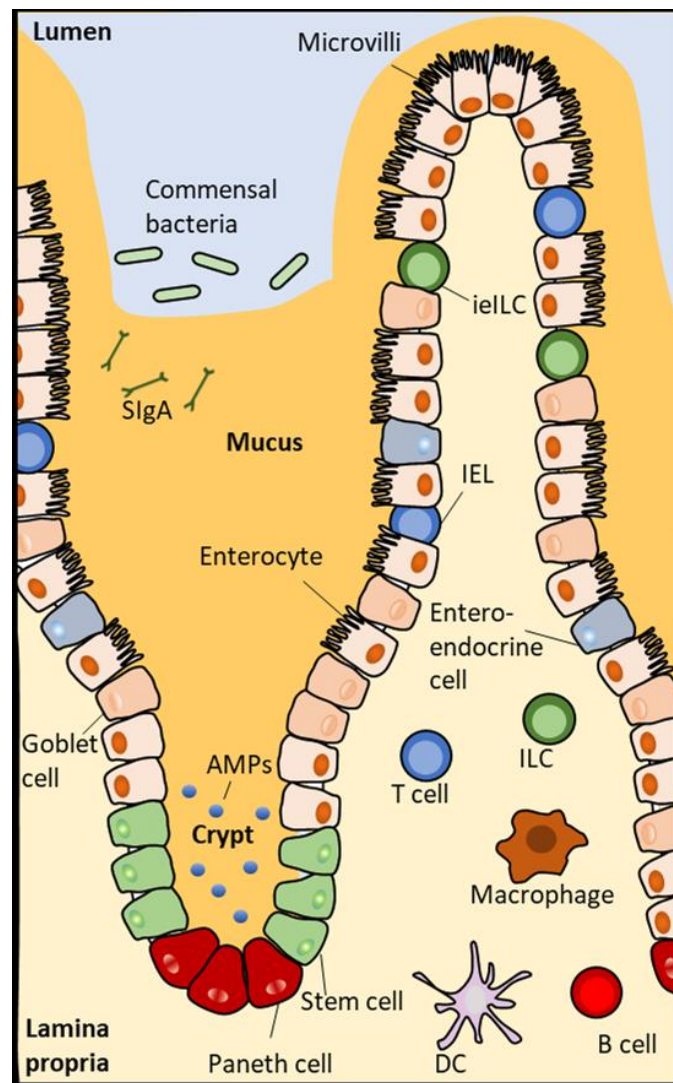
Fig. 3: The intestinal immune system. Intestinal epithelial cells (IEC) including enterocytes, goblet, paneth, enteroendocrine, stem cells, innate immune cells (T, B cells, intraepithelial (ie) innate lymphoid cells (ILC), ie lymphocytes (IEL), macrophages, dendritic cells (DC)) and the commensal bacteria protect the body from external pathogens among others with mucus, secretory immunoglobulin A (SIgA) and antimicrobial peptides (AMP). (adapted from Mowat and Agace, 2014)

The immune cells protecting the intestinal barrier are divided into innate and adaptive immune cells. Innate immune cells are able to react fast to invading pathogens and include monocytes, macrophages, dendritic cells (DCs), granulocytes (eosinophils, neutrophils and basophils), natural killer (NK) cells and innate lymphoid cells (ILCs) (Murphy and Weaver, 2018). The adaptive immune system consists of B and T lymphocytes. Contrary to the innate immune cells, their response is highly specific to the pathogen (Murphy and Weaver, 2018).

All of these barrier protectors have to work together to circumvent altered intestinal microenvironments and intestinal barrier defects that can lead to inflammation (Okumura and Takeda, 2017; Vancamelbeke and Vermeire, 2017).

Inflammation caused by an altered immune response can contribute to cancer development in several ways. Chronic inflammation induced by infectious agents, environmental insults, or autoimmunity can create an environment that is favorable for cancer growth (Greten and Grivennikov, 2019). In addition to direct damage to epithelial cells (Vacante *et al.*, 2020), inflammation can lead to deoxyribonucleic acid (DNA) damage (Kawanishi *et al.*, 2017) and the production of cytokines (Landskron *et al.*, 2014), which promote uncontrolled cell growth and tumor formation. These factors are all induced by immune cells. Hereby, ILCs play a putative role. With their secreted cytokines, they contribute to inflammation and can in turn facilitate tumor growth and progression (Pasquale *et al.*, 2021).
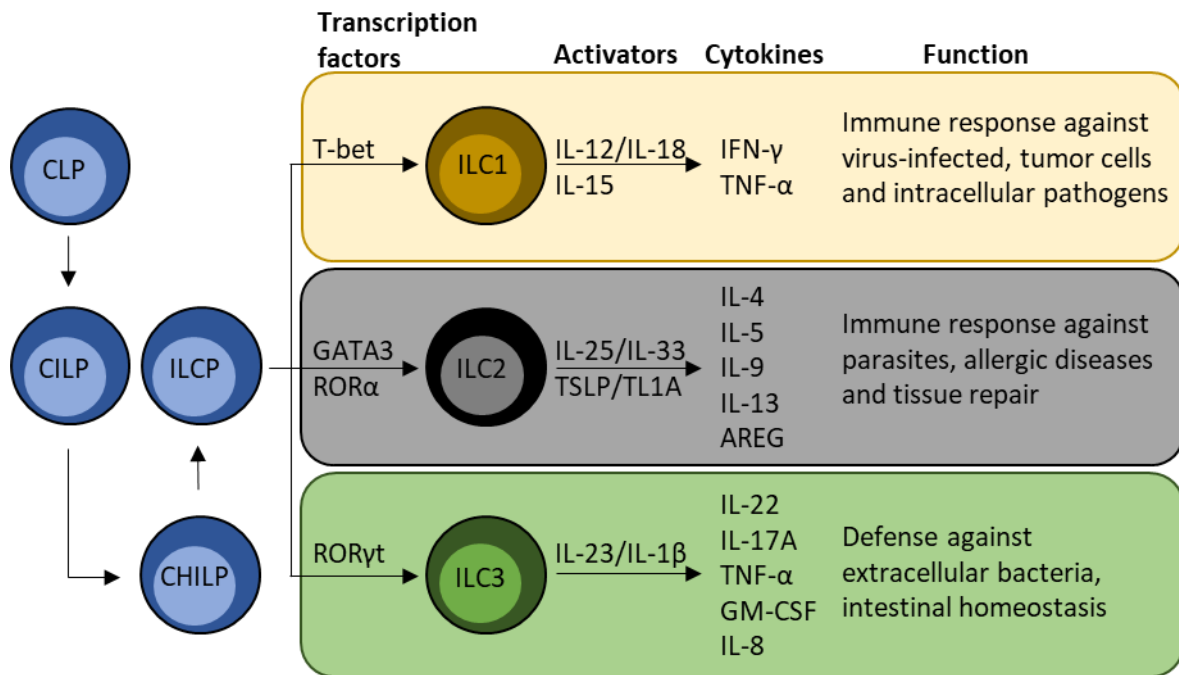
1.3 Innate lymphoid cells and their plasticity

The ILC family is part of the innate immune system and is comprised of several subtypes with distinct functions. These include NK cells, which have cytolytic abilities against virus-infected cells, and were first identified in 1975 (Herberman *et al.*, 1975; Kiessling *et al.*, 1975). In 1997, lymphoid tissue inducer (LTi) cells were identified and found to be involved in the formation of secondary lymphoid organs during fetal development (Mebius *et al.*, 1997). Moreover, helper ILCs, which have three additional subsets, were initially described in 2008 (Cella *et al.*, 2009; Cupedo *et al.*, 2009; Neill *et al.*, 2010; Satoh-Takayama *et al.*, 2008; Spits and Di Santo, 2011). In recent years, the understanding of helper ILCs has advanced through further research and refinement of their concept (Artis and Spits, 2015; Zhong *et al.*, 2018). However, our understanding of helper ILCs is still limited as they are found in smaller numbers and in less accessible tissues compared to other lymphocyte subsets.

Helper ILCs are commonly defined as being lineage (Lin)⁻ cells (excluding myeloid, stem, NK, T and B cells) that express the interleukin (IL)-7 receptor α (Cluster of Differentiation (CD)127) (Spits *et al.*, 2013). They are located in peripheral tissues and abundant at

mucosal sites (Vivier *et al.*, 2018). Thus, ILCs were initially thought to represent "tissue-resident cells", but further evidence has demonstrated that ILCs, much like other immune cells, have the ability to move between and within organs during periods of inflammation (Jacquelot *et al.*, 2022). Their main role is to support early stages of innate immune response via the secretion of cytokines, which plays an important role in the immune response, inflammation, tissue homeostasis and repair (Saez *et al.*, 2021; Vivier *et al.*, 2018). They have a lymphoid lineage, similar to NK, T, and B cells, and are characterized by their secretion of cytokines, similar to those produced by adaptive T cells. Therefore, helper ILCs are commonly referred to as the innate counterpart of T helper (Th) cells (Artis and Spits, 2015; Vivier *et al.*, 2018).

The ILC subsets include group 1 ILCs (ILC1), the innate counterpart of Th1 cells, which function as protection against intracellular pathogens (viruses) and tumors (Vivier *et al.*, 2018), group 2 ILCs (ILC2), which mimic Th2 cells and protect against parasites and allergens (Vivier *et al.*, 2018) as well as group 3 ILCs (ILC3), the counterpart of Th17 cells, which help to prevent the entry of bacteria and fungi and to maintain the epithelial barrier together with DCs and macrophages (Vivier *et al.*, 2018). Although all three groups are considered to arise from the same ID2$^+$ ILC precursor (ILCP), differences in transcription factors usage and dependency, expression of subtype-specific surface molecules, such as tyrosine-protein kinase ckit (CD117) and prostaglandin D2 receptor 2 (CRTH2) and specific cytokine secretion patterns allow for the clear characterization of each subset (Spits *et al.*, 2013) (Fig. 4).

Fig. 4: Development of helper ILCs and their produced cytokines and functions. ILC develop from CLPs (Common lymphoid progenitor), CILPs (Common innate lymphoid progenitor), CHILPs (Common helper ILP) to ILCP (innate lymphoid cell progenitor). The ILC subsets arise from ILCPs and can be differentiated with their transcription factors, cytokines and functions. (adapted from Shi *et al.*, 2022)

ILC1s are a very heterogeneous group as they include NK cells, helper ILC1s and intraepithelial (ie) ILC1s (Cella and Robinette, 2021). Helper or CD127⁺ ILC1s have neither CD117 nor CRTH2 but express the T-box transcription factor *TBX21* (T-bet) and secrete interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) after activation with IL-12, IL-18 and/or IL-15 (Artis and Spits, 2015; Spits *et al.*, 2013). Although they share many characteristics with NK cells, they lack the ability to release granzyme and perforin, do not express eomesodermin (EOMES), and do not exhibit any or only minimal cytotoxic activity. IeILC1s are found within the ie cell surface and are commonly defined as CD127⁻, natural cytotoxicity triggering receptor 2 (NKp44)⁺ and integrin alpha E (CD103)⁺ (Cella and Robinette, 2021). They express T-bet and EOMES and sometimes secrete perforin and granzymes which still makes it difficult to differentiate them from NK cells (Cella and Robinette, 2021).

ILC2s express the transcription factor GATA-binding protein 3 (GATA3) and retinoic acid related orphan receptor (ROR) alpha (Artis and Spits, 2015; Spits *et al.*, 2013; Vivier *et*

al., 2018). Upon stimulation with IL-25, IL-33, thymic stromal lymphopoietin (TSLP) and/or tumor necrosis factor-like cytokine 1A (TL1A) ILC2s secrete IL-5, IL-4, IL-9, IL-13 and/or amphiregulin (AREG) (Artis and Spits, 2015; Spits *et al.*, 2013; Vivier *et al.*, 2018). They are commonly characterized by the expression of CRTH2 and display a high expression of killer cell lectin-like receptor B1 (KLRB1/CD161) and G1 (KLRG1) (Hazenberg and Spits, 2014; Mjösberg *et al.*, 2011).

ILC3s express CD117, depend on RORγt and produce typically IL-17A, IL-22, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-8, IL-2, TNF-α and IFN-γ after stimulation with aryl hydrocarbon receptor (Ahr) ligands or IL-1β and IL-23 (Artis and Spits, 2015; Spits *et al.*, 2013; Vivier *et al.*, 2018). ILC3s can be further subdivided by their NKp44 receptor expression. NKp44+ ILC3s induce the secretion of IL-22 and NKp44- ILC3s produce IL-17A (Artis and Spits, 2015; Hoorweg *et al.*, 2012; Spits *et al.*, 2013; Vivier *et al.*, 2018).

However, circulating NKp44- CD117+ ILCs express low levels of RORγt, do not secrete cytokines and can differentiate into a variety of ILC lineages so that they are better known as ILCPs (Lim *et al.*, 2017). Multipotent ILCPs circulate in peripheral blood and can differentiate dependent on the local microenvironment of the tissues (Lim *et al.*, 2017; Bal *et al.*, 2016). This explains their heterogeneity in several tissues such as the low amount of ILC2s in the gut (Krämer *et al.*, 2017) whereas they are more abundant in the lung (Grove *et al.*, 2016) and skin (Bernink *et al.*, 2019; Mjösberg *et al.*, 2011).

Mature ILC subsets within the tissue can also differentiate according to the local microenvironment and their plasticity is influenced by factors such as Ahr (Qiu *et al.*, 2013), notch ligands (Golub, 2021) and cytokines (Golebski *et al.*, 2019).

In-vitro studies have shown that tonsil NKp44- ILC3s can convert into ILC1s and NKp44+ ILC3s when exposed to IL-2 (Bernink *et al.*, 2013). Human NKp44+ ILC3s can be converted to IFN-γ-producing ILC1-like cells in the presence of a cytokine milieu of IL-12, IL-15, and IL-18 (Fig. 5), as indicated by an upregulation of T-bet and down-regulation of RORγt (Bernink *et al.*, 2015; Cella *et al.*, 2019; Vonarbourg *et al.*, 2010). On the other hand, IL-1β and IL-23 have been shown to reverse the conversion of ILC1s to IL-22-producing NKp44+ ILC3s (Bernink *et al.*, 2015; Cella *et al.*, 2009).

Intermediates of ILC1s and ILC3s have been found in the intestinal mucosa, affirming the conversion (Cella *et al.*, 2019). Moreover, in steady state conditions, ILC3s are increased in the colon, whereas under inflammatory conditions, ILC1s are increased, indicating a conversion from ILC3 to ILC1 (Bernink *et al.*, 2015; Takayama *et al.*, 2010; Lim *et al.*, 2017).
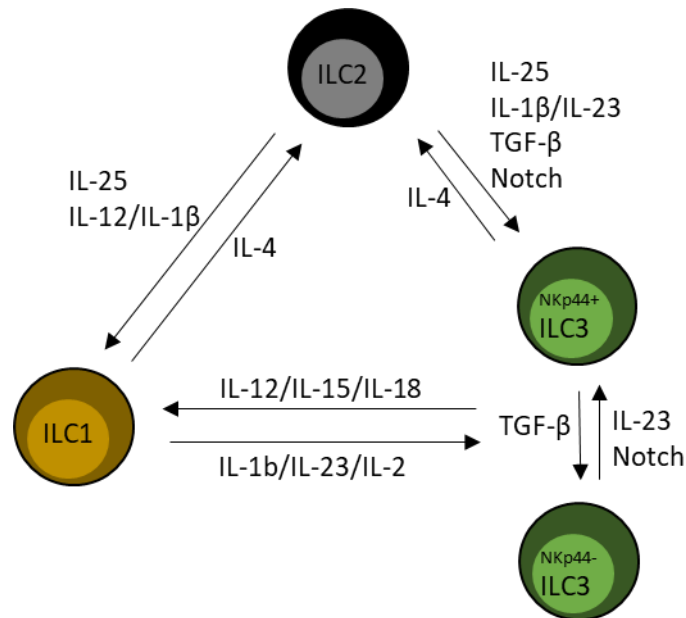
Fig. 5: Plasticity of ILCs. ILC subsets can convert according to specific ligand signaling in all ILC subsets. (adapted from Saez *et al.*, 2021)

The conversion of NKp44$^+$ ILC3s to NKp44$^-$ ILC3s has been established to be transforming growth factor beta 1 (TGF-β1) dependent in mice (Viant *et al.*, 2016). The reversion can be induced by IL-23 and Notch signaling (Klose *et al.*, 2013; Rankin *et al.*, 2013). In addition, changes in the microbiota may also be involved in the conversion of NKp44$^-$ ILC3s to NKp44$^+$ ILC3s.

ILC2s also exhibit plasticity and can convert into ILC1s and ILC3-like cells when exposed to IL-25 (Colonna, 2018; Huang *et al.*, 2015). Additionally, IL-1β, IL-23, and TGF-β have been shown to induce the transformation of ILC2s into ILC3s or ILC3-like cells (Bernink *et al.*, 2019; Golebski *et al.*, 2019), which is dependent on Notch signaling (Zhang *et al.*, 2017) and Ahr (Li *et al.*, 2018). Moreover, both human and mouse ILC2s have been demonstrated to convert into ILC1-like cells which secrete IFN-γ in response to IL-12 and IL-1β (Lim *et al.*, 2016; Ohne *et al.*, 2016; Silver *et al.*, 2016; Colonna, 2018). However, it

remains unclear whether ILC1s and ILC3s can be converted to ILC2s via cytokines such as IL-4 (Bal *et al.*, 2016; Golebski *et al.*, 2019).

The high plasticity of mature helper ILCs enables them to rapidly adapt to changes in the local microenvironment, allowing them to quickly respond to inflammatory signals. Nevertheless, further research is required to comprehend the impact of ILC plasticity in inflammatory disorders and diseases, such as cancer.

1.4 Innate lymphoid cells in intestinal homeostasis and inflammatory diseases

ILCs are crucial for the immune response, inflammatory processes and protection against pathogens at mucosal sites (Saez *et al.*, 2021; Vivier *et al.*, 2018). They are important players in maintaining the intestinal homeostasis, where they increase in frequency from the human upper to the lower GI tract (Krämer *et al.*, 2017). In the upper GI tract, ILC1s are the dominant group, whereas in the ileum and colon ILC3s are more abundant (Krämer *et al.*, 2017; Saez *et al.*, 2021). ILC2s are hardly found in the adult gut (Krämer *et al.*, 2017; Saez *et al.*, 2021). The major function of ILCs is the secretion of cytokines (Marafini *et al.*, 2015). To protect the intestinal barrier against pathogens and tissue damage, ILCs are activated by cytokines and alarmins of epithelial cells and other innate immune cells to maintain the intestinal homeostasis (Ignacio *et al.*, 2017).

ILC1s are known to protect against intracellular pathogens such as viruses and tumors by producing the pro-inflammatory cytokines IFN-γ and TNF-α, thereby triggering an immune response (Fuchs, 2016; Coman *et al.*, 2022). Additionally, they also help in inflamed intestinal tissue by secreting TGF-β1 to expand epithelial crypts and drive intestinal epithelial remodeling (Jowett *et al.*, 2021). Although the interaction of ILC1s with the commensal microbiome is only incompletely understood, data obtained in mouse studies suggest that T-bet is upregulated by the microbiota and important for the interplay between DCs, ILC1s and the microbiota (Klose *et al.*, 2013; Powell *et al.*, 2012; Saez *et al.*, 2021). ILC1s also have the capability to protect against bacteria such as *Clostridium difficile* and *Toxoplasma gondii* to maintain tissue homeostasis (Gury-BenAri *et al.*, 2016).

Despite their low frequency at intestinal mucosal sites in humans, ILC2s have a variety of functions in mice. Their most prominent function is protecting the intestines against helminths by secreting cytokines such as IL-13 (Moltke *et al.*, 2016). This cytokine is produced in response to IL-25 secreted by tuft cells in the epithelial layer, which results in increased goblet and tuft cells and the removal of parasites through mucus (Moltke *et al.*, 2016; Gerbe *et al.*, 2016; Schneider *et al.*, 2018). Additionally, ILC2s produce and secrete AREG, which can trigger epidermal growth factor receptor (EGFR) signaling of regulatory T (Treg) cells to enhance their immunosuppressive potential (Zaiss *et al.*, 2013; Luo and Villablanca, 2021). Together with the enteric nervous system, ILC2s also play a role in achieving intestinal immune homeostasis (Xu *et al.*, 2019).

ILC3s are activated by IL-23 and IL-1β secreted by DCs and macrophages in the LP, which triggers the secretion of IL-22, IL-17A and GM-CSF. IL-22-producing ILC3s protect intestinal stem cells from tissue damage by activating stem cell proliferation, thereby increasing the epithelial barrier function (Zeng *et al.*, 2019; Sonnenberg *et al.*, 2012; Hanash *et al.*, 2012). They prevent the dissemination of commensal bacteria into the intestinal lumen which normally results in systemic inflammation (Zeng *et al.*, 2019; Sonnenberg *et al.*, 2012; Hanash *et al.*, 2012). To further help maintaining intestinal homeostasis, their cytokines activate paneth cells to produce AMPs which kill pathogens (Guo *et al.*, 2014). Through the expression of their histocompatibility complex class II, they interact with CD4^{+} T cells to regulate the adaptive immune response and the commensal bacteria (Hepworth *et al.*, 2013). IL-17A produced by ILC3s protects the intestinal barrier by regulating tight junctions (Lee *et al.*, 2015). Additionally, IL-17A recruits neutrophils to inflammatory sites to promote the immune response (Flannigan *et al.*, 2017).

The dysregulation of intestinal ILCs has been linked to the development of several diseases such as inflammatory bowel diseases (IBD) (Forkel and Mjösberg, 2016; Chuang *et al.*, 2022; Saez *et al.*, 2021), autoimmune diseases (celiac disease) (Marafini *et al.*, 2015) and food allergies (Pasha *et al.*, 2019). During chronic intestinal inflammation, microbial dysbiosis can lead to an excessive production of pro-inflammatory cytokines such as IL-12, IL-18, IL-23 and IL-1β secreted by inflammatory DCs and macrophages (Geremia and Arancibia-Cárcamo, 2017). This in turn activates and increases subsets of ILCs which secrete high levels of IFN-γ and IL-17A (Geremia and Arancibia-Cárcamo,

2017). The high amount of these ILC-derived cytokines stimulates the recruitment of additional inflammatory cells such as neutrophils and exacerbates the chronic nature of inflammation and tissue damage. Increased levels of IFN-γ and TNF-α secreted by ILC1s is considered to increase the permeability of the intestinal epithelial cell barrier, thus allowing bacterial entry and further promoting inflammation (Ganal-Vonarburg and Duerr, 2020; Bernink *et al.*, 2013). A lack of protection by decreased ILC3s in the colon of IBD patients may contribute to the development of IBD (Ganal-Vonarburg and Duerr, 2020; Bernink *et al.*, 2013). High levels of IL-33 have been shown to be a contributing factor to the development of food allergies, as it promotes the expansion and activation of ILC2s, which in turn produce high concentrations of IL-4 and suppress Treg cell activity (Noval Rivas *et al.*, 2015).

Also in other tissue compartments such as the pulmonary system (Grove *et al.*, 2016; Hsu *et al.*, 2021) and the skin (Zhou *et al.*, 2020), ILCs have been shown to play a major role in inflammatory diseases, albeit with different functions that depend on the local microenvironment.

This highlights the complex nature of ILCs which can demonstrate plasticity depending on the particular microenvironments within the body. This plasticity can give rise to either protective or pathogenic functions during inflammatory diseases. Furthermore, inflammatory states, such as in patients with IBD, have been linked to an increased risk of developing CRC. It is likely that the tumor microenvironment (TME) also contributes to modulating ILC plasticity and function, potentially leading to tumor progression.

1.5   ILCs in cancers

The initiation of cancer is induced by sporadic (external factors) or hereditary mutations in tumor suppressor genes leading to abnormal cell growth. The cellular and molecular elements surrounding the tumor, known as the TME, are essential for the growth and spread of cancer. The TME is a complex system consisting of tumor cells, stromal cells, blood vessels, immune cells and extracellular matrix (Anderson and Simon, 2020). In the TME, the cells communicate with each other through secretion of cytokines in a dysregulated manner (Elsawa *et al.*, 2011; Landskron *et al.*, 2014; Borowczak *et al.*, 2022;

Li *et al.*, 2020a). Immune cells can detect these tissue changes and eliminate abnormal cells in a process called immunosurveillance (Warner *et al.*, 2022). However, nowadays the process is rather divided, with immune cells acting as pro- or anti-tumor effectors that either promote or inhibit tumor progression (Elsawa *et al.*, 2011; Landskron *et al.*, 2014; Borowczak *et al.*, 2022; Li *et al.*, 2020a). The role of T and NK cells in tumor suppression and their anti-tumorigenic effects are well established; in contrast, less is known about the role of ILCs in this regard and cannot be definitively classified as either pro- or anti-tumorigenic. This may be due to their ability to rapidly adapt to changing conditions, such as the local cytokine microenvironment, and their ability to alter their phenotype and cytokine production in response to changes in the TME. However, recent research has emphasize the significant role of ILCs in determining the TME and affecting the genesis and spread of cancer (Atreya *et al.*, 2019; Bruchard and Spits, 2022; Wang *et al.*, 2020a).

In order to effectively combat tumor growth and eliminate cancer cells, immune cells must migrate to the tumor. Studies have indirectly demonstrated that ILCs are able to migrate to tumors, as increased levels of ILCs have been detected in peripheral blood of cancer patients (Loyon *et al.*, 2019; Bie *et al.*, 2014; Weerdt *et al.*, 2016). It is also likely that ILCs expand or convert in the TME as increased numbers of ILC subsets have been observed in several cancers such as breast and GI cancer (Salimi *et al.*, 2018), lung cancer (Carrega *et al.*, 2015) hepatocellular carcinoma (HCC) (He *et al.*, 2022) and CRC (Carrega *et al.*, 2020; Goc *et al.*, 2021). The underlying mechanism by which ILCs expand in the TME remains elusive (Jacquelot *et al.*, 2022). However, the increased ILCs may be either present due to the recruitment of ILCs to the tumor, or the expansion or conversion in another subset within the tumor which has yet to be explored (Ducimetière *et al.*, 2019).

Normally, ILC1s would rather be anti-tumorigenic eliminating cancer cells with IFN-γ and TNF-α but in several carcinomas such as HCC, ILC1s exhibit an impaired immunosurveillance function due to decreased ILC1s (He *et al.*, 2022). In other cancers, their frequency were found to be increased such as in peripheral blood mononuclear cells (PBMCs) and tumor-infiltrated lymph nodes of melanoma patients (Ercolano *et al.*, 2020) and CRC tissues (Carrega *et al.*, 2020; Qi *et al.*, 2021; Goc *et al.*, 2021) but exhibited an impaired cytokine secretion ability. This impaired immunosurveillance function is thought to contribute to the observed pro-tumorigenic role of ILC1s. In CRC, studies have reported

a conversion of ILC3s into ILC1s (Cella *et al.*, 2019; Goc *et al.*, 2021). Furthermore, Goc *et al.* (2021) demonstrated that the decrease in ILC3s reduced the interaction with T cells and promoted tumor development. This highlights the plasticity of ILCs in the TME. However, depending on the cancer and the TME, ILC1s can also be anti-tumorigenic (Dadi *et al.*, 2016; Kansler *et al.*, 2022). ILC1-mediated cytotoxicity against tumor cells has been demonstrated in chromophobe renal cell carcinoma, and it has been found that this cytotoxicity is reliant on the production of IL-15 by the tumor cells (Kansler *et al.*, 2022). In a mouse model of mammary tumors, ILC1s infiltrating tumors have been found to exhibit direct tumor-killing activity by producing high levels of granzyme B (Dadi *et al.*, 2016).

In certain types of cancer, such as breast, gastric cancer (Salimi *et al.*, 2018), CRC (Jou *et al.*, 2022), PBMCs of acute promyelocytic leukemia patients (Trabanelli *et al.*, 2017) and non-small cell lung cancer (Shen *et al.*, 2021), ILC2s have been found to increase in number, and their presence has been linked to poor prognosis and survival. ILC2s together with myeloid-derived suppressor cells have also been shown to impair the anti-tumor response (Jou *et al.*, 2022). Accordingly, ILC2 have been found to suppress NK cell-mediated anti-tumor immunity, leading to increased lung metastases and mortality (Schuijs *et al.*, 2020). On the other hand, ILC2s have been found to possess anti-tumor functions through their interaction with eosinophils (Ikutani *et al.*, 2012; Jacquelot *et al.*, 2021), and CD8$^+$ T cells. For instance, in a mouse model of melanoma, ILC2s were found to inhibit tumor growth by recruiting CD8$^+$ T cells via IL-33, which contributed to tumor suppression (Okuyama *et al.*, 2022).

As mentioned earlier, ILC3s have pro-tumorigenic effects in CRC by inhibiting anti-tumor T cell response (Carrega *et al.*, 2020; Goc *et al.*, 2021). Additionally, squamous cell carcinoma (SCC) is associated with a conversion of ILC1s to ILC3s leading to tumor progression (Koh *et al.*, 2019). Similarly, in HCC, a conversion of ILC1s to NKp44$^-$ ILC3s has been reported and linked to the development of HCC in mice (Liu *et al.*, 2019). Moreover, in a mouse model of IBD, it was found that IL-22-producing ILC3s promote bacteria-induced CRC (Kirchberger *et al.*, 2013). However, ILC3s have also been shown to have anti-tumorigenic effects. For instance, in a mouse model of skin cancer, IL-12 initiated local anti-tumor immunity by stimulating ILC3s (Eisenring *et al.*, 2010).

Furthermore, in lung cancer, ILC3s have been demonstrated to migrate to tumors, inhibit tumor growth via chemokine ligand 20 and IL-1β which leads to the recruitment of anti-tumor immune cells (Bruchard *et al.*, 2022) and contribute to the formation of protective tumor-associated tertiary lymphoid structures (Carrega *et al.*, 2015).

Thus, the exact role of ILCs in tumorigenesis is likely to be complex and may vary depending on the specific type of cancer, the stage of disease, and the tumor microenvironment. Further research is needed to fully understand the role of ILCs in cancer and to develop effective immunotherapies targeting these cells.

Unfortunately, there is not much known regarding the phenotype and function of ILCs in upper GI cancers and even less is known about their role in hereditary GI cancer syndromes such as FAP. Although some mouse data has indicated the importance of ILCs in upper intestinal adenoma formation (Chen *et al.*, 2019b), and IL-17A production in a FAP mouse model (APC$^{min/+}$ mice) has been suggested to promote tumor growth (Chae *et al.*, 2010; Chae and Bothwell, 2015), this increase in IL-17A production was not observed by another group (Agüera-González *et al.*, 2017).

1.6 Aim

The mechanisms underlying the development of duodenal adenomas in FAP patients are still incompletely understood. However, the lack of a clear genotype-phenotype correlation indicates that factors other than the genotype play a role here. Given the increasing body of evidence suggesting a link between the local immune system and various aspects of tumor development, progression, and treatment outcomes, this study aimed to thoroughly characterize the immune cell infiltrate in the duodenum of patients with FAP and to determine the underlying regulatory mechanisms with the overall goal to explore the potential role of the duodenal immune response in the development of duodenal adenomas in FAP patients.

2. Material and methods

2.1 Material

2.1.1 Devices

Tab. 2: Used devices for sample processing and data analysis.

Device	Manufacturer
Centrifuge 5810R	Eppendorf AG
CO_2-Incubator Forma	Thermo Fisher Scientific
FACSAria™ Fusion	BD Biosciences
FACSCanto™ II	BD Biosciences
Light microscope DM IL	Leica Microsystems GmbH
LightCycler® 96 Real-Time PCR System	Roche
LSR Fortessa™	BD Biosciences
Multipipette plus	Eppendorf AG
NanoDrop™ 1000	Thermo Fisher Scientific
NovaSeq 6000 sequencer	Illumina
pH-Meter Portamess 911	Knick Elektronische Messgeräte GmbH
Pipettes (1000, 200, 100, 10 µL)	Eppendorf AG
Pipetus®	Hirschmann Laborgeräte GmbH
Rotator stator homogenizer	Bandelin electronic
Safety cabinet Gelaire	Flow Laboratories GmbH
sterile workbench	Renggli AG
TapeStation System 4200	Agilent
Vortex-Genie 2	Scientific Industries
Water bath	Köttermann Labortechnik

2.1.2 Consumables

Tab. 3: Consumables used in this study.

Material	Manufacturer
1.5 ml reaction vials	Sarstedt AG
70 µm Nylon cell strainer	Corning®

Cell culture flasks (25, 75 cm^2)	Greiner Bio-One
Cell scraper	Sarstedt AG
Cell culture plates CellStar® (6, 12, 24, 48, 96 Well)	Greiner Bio-One
CellStar® tubes (50, 15 mL)	Greiner Bio-One
Cryovials CryoPure	Sarstedt AG
FACS tubes	Sarstedt AG
Nalgene® disposable filtration system	Thermo Fisher Scientific
Pasteur pipettes	Brand®
Pipette tips (1000, 200, 100, 10 µL)	Greiner Bio-One
Pipettes CellStar® for Pipetus®	Greiner Bio-One
96-well PCR microplate	STARLAB International GmbH

2.1.3   Reagents

Tab. 4: Reagents used in this study.

	Cat. Nr.	Manufacturer
2-Mercaptoethanol	M6250-100ML	Sigma Aldrich®
Antibiotic-Antimycotic	15240062	Gibco™
Advanced DMEM/F12	12634-010	Gibco™
Ascorbic acid	A1300000	European Pharmacopoeia
Bovine serum albumin (BSA)	A9418	Sigma Aldrich®
Brefeldin A (BFA)	BML-G405	Enzo Life Sciences
Ca^{++}/Mg^{++}-free Hanks Balanced Salt Solution (10x HBSS)	21765029	Gibco™
Cell Recovery Solution	354253	Corning™
Collagenase Type IV	LS004189	Worthington®
DMEM	41965-039	Gibco™
Dimethyl sulfoxide (DMSO)	A3672	AppliChem GmbH
Dithiothreitol (DTT)	10708984001	Sigma Aldrich®
10x DPBS (without Ca^{++}/Mg^{++})	P04-53500	PANBiotech
Ethanol	A1613	AppliChem GmbH

ethylenediaminetetraacetic acid (EDTA)	A3145	AppliChem GmbH
Fetal Bovine Serum (FBS)		Sigma Aldrich®
Gentamicin	G2023	USBiological
Gentle Cell Dissociation Reagent (GCDR)	100-0485	STEMCELL™ Technologies
Goat serum	S26	Sigma-Aldrich®
Ham's F-12 Nutrient Mix	21765029	Gibco™
HEPES	90909C	Sigma-Aldrich®
Hoechst 33342 Fluorescent Stain	62249	Thermo Fisher Scientific
HS-Nuclease	GE-NUC10700	MoBiTec
Human AB serum	H3667-100ML	Sigma Aldrich®
Human serum albumin (HSA)	001052-31826	CSL Behring
IntestiCult™ Organoid Growth Medium (OGM) (human)	6010	STEMCELL™ Technologies
Ionomycin	9995	Cell Signaling Technology
Matrigel® Matrix	356237	Corning™
N-acetyl cysteine (NAC)	A7250	Sigma-Aldrich®
Pancoll, human	P04-601000	PanBiotec™
Paraformaldehyde (PFA)	158127	Merck KGaA
Penicillin-Streptomycin (P/S)	P06-07050	PanBiotec™
Phorbol-12-myristat-13-acetat (PMA)	9905	Cell Signaling Technology
Phosphate-buffered saline (PBS)	18912014	Gibco™
Prolong™ Gold antifade reagent	P36930	Invitrogen™
RPMI-1640	21875034	Gibco™
Sodium azide	26628-22-8	Merck KGaA
Sodium selenite	S5261-10G	Sigma-Aldrich®, USA
Triton-X	9036-19-5	Merck KGaA
TrueBlack® Lipofuscin	23007	Biotium

2.1.4   Antibodies

Tab. 5: List of antibodies used for flow cytometric analysis.

Antigen	Conjugate	Clone	Cat.-Nr.	Manufacturer
CD103	AF700	Ber-ACT8	NBP1-9756-4AF700	Novus™
CD117 (ckit)	PE/Vio615	REA787	130-111-598	Milteny Biotec
CD117 (c-kit)	PE/Cy7	104D2	313212	BioLegend®
CD123	FITC	6H6	306014	BioLegend®
CD127 (IL-7Ra)	BV605	A019D5	351334	BioLegend®
CD127 (IL-7Ra)	BUV737	HIL-7R-M21	612794	BD Biosciences
CD14	FITC	M5E2	301804	BioLegend®
CD161	APC/Cy7	HP-3G10	339928	BioLegend®
CD19	FITC	HIB19	302206	BioLegend®
CD1a	FITC	HI149	300104	BioLegend®
CD20	FITC	2H7	302304	BioLegend®
CD200R	BV421	OX-108	329314	BioLegend®
CD294 (CRTH2)	BV711	BM16	350124	BioLegend®
CD294 (CRTH2)	PerCP-Cy5.5	BM16	350116	BioLegend®
CD294 (CRTH2)	BV421	BM16	562992	BD Biosciences
CD3	FITC	UCGT1	300406	BioLegend®
CD303 (BDCA-2)	FITC	AC144	130-113-192	Milteny Biotec
CD336 (NKp44)	BV786	P44-8	744304	BD Biosciences
CD336 (NKp44)	APC	P44-8	325109	BioLegend®
CD336 (NKp44)	PerCP-Cy5.5	P44-8	325114	BioLegend®
CD34	FITC	581	343504	BioLegend®
CD4	FITC	OKT4	317408	BioLegend®
CD4	BV805	SK3	612887	BD Biosciences
CD4	PE-Cy7	RPA-T4	300512	BioLegend®
CD45	BUV395	HI30	563792	BD Biosciences
CD45	BV805	HI30	564914	BD Biosciences

CD49a	PerCP/eFluor710	TS2/7	46-9490-42	eBioscience™
CD5	FITC	UCHT2	300606	BioLegend®
CD5	BV805	L17F12	748492	BD Biosciences
CD56	BUV563	NCAM16.2	612928	BD Biosciences
CD8a	FITC	REA734	130-110-677	Milteyi Biotec
CD94	FITC	DX22	305504	BioLegend®
CD94	BV737	HP-3D9	748787	BD Biosciences
CD94	FITC	HP-3D9	555888	BD Biosciences
CD94	APC-Vio770	REA113	130-101-146	Milteyi Biotec
EOMES	eFluor660	WD1928	50-4877-42	eBioscience™
FcεR1α	FITC	AER-37 (CRA-1)	334608	BioLegend®
FCR Block			130-059-901	Milteny Biotec
GATA3	BUV395	L50-823	565448	BD Biosciences
IFN-y	PE/Dazzle 594	4S.B3	502545	BioLegend®
IFN-y	BV421	4S.B3	502532	BioLegend®
IL-13	PE	JES10-5A2	501903	BioLegend®
IL-17A	PerCP-Cy5.5	BL168	512314	BioLegend®
IL-17A	PE	BL168	512306	BioLegend®
IL-2	BV650	MQ1-17H12	500334	BioLegend®
IL-22	APC	IL22JOP	17-7222-82	Invitrogen™
IL-8	PE/Cy7	E8N1	511416	BioLegend®
IL-8	PE	E8N1	511408	BioLegend®
IL1R1	PE		FAB269P-100	R&D Systems®
KLRG1 (MAFA)	BV421	SA231A2	367706	BioLegend®
NKp80	PE/Vio770	4A4.D10	130-105-068	Milteny Biotec
NKp80	FITC	4A4.D10	130-094-843	Milteny Biotec
NKp80	APC/Vio770	REA845	130-112-593	Milteny Biotec
NKp80	PE	REA845	130-112-590	Milteny Biotec

RORγt	PE	AFKJS-9	12-6988-82	eBioscience™	
RORγt	BV421	Q21-559	563282	BD Biosciences	
T-bet	BV711	O4-46	563320	BD Biosciences	
TCRαβ	FITC	IP26	306706	BioLegend®	
TCRγδ	FITC	B1	331208	BioLegend®	
TNF-α	BV785	MAb11	502948	BioLegend®	
Isotype controls:					
	APC	eBR2a	17-4321-81	eBioscience™	
	BV421	MOPC-21	400158	BioLegend®	
	BV711	X40	563044	BD Biosciences	
	BUV395	X40	563547	BD Biosciences	
	PE	eBR2a	12-4321-80	eBioscience™	

Tab. 6: List of used antibodies for immunofluorescence and blocking.

Antibody	Host	Class	Conc. / Dilution	Cat. Nr.	Manufacturer
anti-Epcam	mouse	Monoclonal	5 µg/ml	MA1-10196	Invitrogen™
anti-Duox2	rabbit	Polyclonal	5 µg/ml	NB110-61576SS	Novus™Biologicals
anti-Lysozyme	rabbit	Polyclonal	5 µg/ml	NBP2-61118	Novus™Biologicals
anti-Muc2	rabbit	Monoclonal	5 µg/ml	MA5-32654	Thermo Fisher Scientific
anti-IL-17A	goat	Polyclonal	2 µg/ml	AF-317-SP	R&D Systems®
anti-NKp46	goat	Polyclonal	2 µg/ml	AF-1850	R&D Systems®
anti-mouse AF488	donkey	Polyclonal	4 µg/ml	ab150105	abcam
anti-rabbit AF555	donkey	Polyclonal	4 µg/ml	ab150070	abcam

2.1.5   Oligonucleotides

Tab. 7: List of primers used for qRT-PCR. (Fw = forward, Rev = reverse)

Gene	Fw Primer 5'-3' Seq	Rev Primer 5'-3' Seq
CAPN8	GACTTCCAGGAGAACTATGCGG	TCCGAGTGTAGGAAGAGCAGCT
CDH3	CAGGTGCTGAACATCACGGACA	CTTCAGGGACAAGACCACTGTG
CEMIP	ACCGAGCACATTCCAACTACCG	GGCAGAGATGATTGAGAGGAACG
DL1	GATTCTCCTGATGACCTCGCA	TCCGTAGTAGTGTTCGTCACA
DL4	GTCTCCACGCCGGTATTGG	CAGGTGAAATTGAAGGGCAGT
DUOX2	CTGGGTCCATCGGGCAATC	GTCGGCGTAATTGGCTGGTA
DUOXA2	AACGGCGTACTGCCTTTTTAC	GAGAAGAACTCTCACCAACCAAA
EEF1A1	CCG TTC TTC CAC CAC TGA TT	CTT TGG GTC GCT TTG CTG TT
IFNg	GTATTGCTTTGCGTTGGACA	GAGTGTCGAGACCATCAAGGA
IL12A	TGCCTTCACCACTCCCAAAACC	CAATCTCTTCAGAAGTGCAAGGG
IL13	TTTCGCGAGGGACAGTTC	CAAGGGGAAGGCTGAGGT
IL15	CCATCCAGTGCTACTTGTGTTTACTT	CCAGTTGGCTTCTGTTTTAGGAA
IL17A	CGG ACT GTG ATG GTC AAC CTG A	GCA CTT TGC CTC CCA GAT CAC A
IL18	ACTGGTTCAGCAGCCATCTT	TGCAGTCTACACAGCTTCGG
IL1B	GAA GCT GAT GGC CCT AAA CA	AAG CCC TTG CTG TAG TGG TG
IL2	CCAAGAAGGCCACAGAACTGA	AATGGTTGCTGTCTCATCAGC
IL22	CTC TGG ATA TGC AGG TCA TCA C	AGT GCT GTT CCC TCA ATC TG
IL23A	CTC AGG GAC AAC AGT CAG TTC	ACA GGG CTA TCA GGG AGC A
IL33	AAG GCA AAG CAC TCC ACA GT	CAA AGA AGT TTG CCC CAT GT
IL4	CCAACTGCTTCCCCCTCTG	TCTGTTACGGTCAACTCGGTG
IL5	TGGAGCTGCCTACGTGTATG	TTCGATGAGTAGAAAGCAGTGC
IL8	AAA TTT GGG GTG AAA GGG TT	TCC TGA TTT CTG CAG CTC TGT
KI67	TCCTTTGGTGGGCACCTAAGACCTG	TGATGGTTGAGGTCGTTCCTTGATG

KRT7	TCCGCGAGGTCACCATTAAC	GCTCTGTCAACTCCGTCTCAT
LGR5	CCTGCTTGACTTTGAGGAAGAC C	CCAGCCATCAAGCAGGTGTTCA
LYZ	TCAATAGCCGCTACTGGTGTA	ATCACGGACAACCCTCTTTGC
MUC-2	GGAGATCACCAATGACTGCGA	GAATCGTTGTGGTCACCCTTG
S100P	CTCAAGGTGCTGATGGAGAAGG	GAACTCACTGAAGTCCACCTGG
TGFb1	TGGCGATACCTCAGCAACC	CTCGTGGATCCACTTCCAG
TL1A	CACATACCTGCTTGTCAGCC	TGTGAAGGTGCAAACTCCTG
TNFa	CTCTTCTGCCTGCTGCACTTTG	ATGGGCTACAGGCTTGTCACTC
TSLP	ATG TTC GCC ATG AAA ACT AAG GC	GCG ACG CCA CAA TCC TTG TA
TSPAN1	TGCTGTGGTCGCCTTGGTGTAC	TGGTGAAGCCACAGCACTTGAG

2.1.6   Kits

Tab. 8: List of used Kits.

Kits	Manufacturer
Blue S' Green qPCR Kit	Biozym®
Cytofix/Cytoperm™ Fixation/Permabilization Kit	BD Bioscience
eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set	Invitrogen™
GeneJet RNA Purification Kit	Thermo Fisher Scientific
LEGENDplex™ Human Th Cytokine Panel kit	Biolegend®
QuantiTect® Reverse Transcription Kit	Qiagen
Zombie Aqua™ Fixable Viability Kit	Biolegend®

2.1.7   Cytokines

Tab. 9: Used human recombinant cytokines.

Cytokines	Cat. Nr.	Manufacturer
IL-1β	11340015	Immunotools
IL-2	130-097-743	Miltenyi Biotec
IL-22	11340223	Immunotools
IL-23	11340233	Immunotools

IL-17A	570504	Biolegend®
IL-7	130-095-367	Miltenyi Biotec
IL-8	11349084	Immunotools
TNF-α	11343015	Immunotools

2.1.8 Buffer and media

Tab. 10: Preparation of used buffers and cell culture media.

Buffer/Medium	Component
10x NAC	1.25 g NAC in 1x HBSS (pH 7.4)
Antibody buffer	1 % BSA, 0.05 % Triton-X, 0.04 % sodium acid in 1x DPBS
Blocking buffer	5 % goat serum, 1 % BSA, 0.1 % Triton-X, 0.04 % sodium acid in 1x DPBS
Cell culture Medium (cRPMI)	10 % FBS, 1 % P/S in RPMI1640
Digestion Medium	100 U/ml Collagenase Type IV, 25 U/ml HS-Nuclease in cRPMI
DMEM + 1 % BSA	15 mM HEPES, 1 % BSA in Advanced DMEM/F12
Freezing Medium	10 % DMSO in cRPMI
NAP buffer	7.44 g EDTA, 7.35 g sodium citrate trisodium salt dihydrate, 700 g ammonium sulfate in H_2O (pH 5.2)
OP9 Medium	10 % FBS, 1 % P/S in DMEM
OP9 Differentiation Medium	10 % human AB serum, 1 % Antibiotic-antimycotic, 20 mg/ml ascorbic acid, 0.05 mg/ml sodium selenite, 24 mM 2-mercaptoethanol in Ham's F12 Nutrient Mix
Organoid Growth Medium (OGM)	50 µg/ml gentamycin in OGM Human Basal Medium with organoid supplement (1:1)
Pre-Digestion Medium	154 µg/ml DTT, 5 mM EDTA, 0.25 % 1x NAC, 1 % P/S in 1x HBSS
Thawing Medium	25 U/ml HS-Nuclease in cRPMI

2.1.9 OP9/OP9-DL1/OP9-DL4 stromal cells

OP9, OP9 expressing Notch ligand Delta-like (DL) 1 and OP9 expressing Notch ligand DL4 were kindly provided by Prof. Dr. Marcus Uhrberg, Prof. Dr. Juan Carlos Zuniga-Pflucker and Prof. Dr. Diefenbach (Mohtashami *et al.*, 2013).

2.1.10 Human Samples

For this study, 20 - 35 ml of heparinized whole blood was taken from FAP and non-FAP patients by the Gastroenterology-Hepatology outpatient clinic and the Immunology outpatient clinic of the Medical Clinic and Polyclinic I of the University Hospital Bonn. All subjects underwent esophagogastroduodenoscopy and colonoscopy for adenoma and cancer screenings. Duodenal and colonic normal and adenomatous mucosa from FAP patients were collected (Tab.11). Normal mucosa was obtained from non-FAP patients who served as controls. Tonsils were obtained from Medical Clinic and Polyclinic for Otolaryngology after tonsillectomy. All patients signed an informed consent form for the use of the patient material for scientific testing purposes. The study had been approved by the local ethics committee of the Medical Faculty, University of Bonn (079/13, 040/16, and 493/20). All research was performed in accordance with both the Declarations of Helsinki and Istanbul.

Tab. 11: Patient characteristic.

	non-FAP patients	**FAP patients**
Duodenal tissue:	35	90
Spigelman stage (0-IV):		
SS 0		11
SS I		19
SS II		19
SS III		27
SS IV		12
Duodenal Adenoma		22
Colonic tissue	20	30
Colonic Adenoma		19
PBMCs	15	37
Tonsil	3	

2.2   Methods

2.2.1   Isolation of intestinal lymphocytes

According to established protocols, intestinal lymphocyte isolation was carried out from macroscopically normal and adenomatous intestinal tissue samples obtained during routine endoscopy (Bernink *et al.*, 2013; Krämer *et al.*, 2017). The tissue samples were treated with pre-digestion medium (Tab. 10) for 45 min at 37°C to remove epithelial tight junctions and mucus. After centrifugation (3 min, 50 g), the supernatant was collected, and tissue samples were then treated with digestion medium (Tab. 10) at 37°C for 60 min. After being filtered through a 70 µm nylon cell strainer using a cell scraper, the intraepithelial cells from the collected supernatant were combined with the lamina propria cells. The samples were then pre-frozen in in a polystyrene tray at -80°C using 0.5 ml freezing medium (Tab. 10) and cooled down at a rate of 1°C/min. After 24 h, the cells were moved to -150°C for long-term preservation until used for phenotypic and functional flow cytometric analysis or ILC sorting on OP9 feeder cells.

2.2.2   Isolation of PBMCs

Using density gradient centrifugation, peripheral blood mononuclear cells (PBMCs) were isolated from fresh, heparinized blood. Heparinized, diluted whole blood was applied on top of Pancoll. The interphase containing lymphocytes was collected after centrifugation at 1000 g for 15 min without a pause. The lymphocytes were PBS washed, then pelleted at 500 g for 10 min with a pause. The cells were frozen as biopsies and used for phenotypic and functional flow cytometric analysis.

2.2.3   Isolation of tonsil lymphocytes

For isolation of tonsil lymphocytes, tonsils were cut into small pieces and squeezed through a stainless-steel mesh. Single lymphoid cells were isolated using Pancoll gradient

centrifugation as PBMCs. Similar to intestinal lymphocytes, the cells were frozen and used for ILC sorting for further experiments with OP9 stromal cells.

2.2.4 Thawing of cells

Thawing of lymphocytes was performed at 37°C in the water bath for 1-2 min until the ice crystals were dissolved. The thawed cell suspension was added to a 15 ml falcon and thawing medium was gradually added in drips until the dimethyl sulfoxide (DMSO) concentration was at least diluted by <0.1 %. The cell suspension was centrifuged (350 g, 10 min) to remove the freezing and thawing medium. Cells were maintained in cRPMI (Tab. 10) for further experiments.

2.2.5 Cell stimulation for intracellular staining

To stimulate lymphocytes of intestinal biopsies or PBMCs, approx. 200000 cells were incubated in 48 well plates in 500 µl cRPMI (Tab. 10). PMA (50 ng/ml) and Ionomycin (1 µg/ml) (P/I) were appended to the culture to measure their overall functional capacity. After 1 h, Brefeldin A (BFA) was added to perform a flow cytometric analysis of cytokine production. Unstimulated cells were used as controls.

2.2.6 Cultivation of OP9, OP9-DL1 and OP9-DL4 feeder cells

OP9 stromal cells, OP9 expressing Notch ligand delta-like (DL) 1 or DL4 (OP9-DL1, OP9-DL4) derived from mouse bone marrow stromal cells were used as feeder cells. They were kindly provided by Prof. Dr. Marcus Uhrberg and Prof. Dr. Juan Carlos Zuniga-Pflucker and Prof. Dr. Andreas Diefenbach (Mohtashami *et al.*, 2013). They were cultured in OP9 medium (Tab. 10) at 37 °C and 5 % carbon dioxide (CO_2).

2.2.7   Co-incubation of ILC3s and OP9-DL4

To co-incubate sorted ILCs with OP9 feeder cells, the OP9 cells were seeded in 24 well plates (10000 cells/well) for bulk culture, irradiated (25 Gray) two days prior to the addition of sorted NKp44- ILC3s and recovered in differentiation medium (Tab. 10) (Cichocki and Miller, 2010). Sorted duodenal or tonsil NKp44- ILC3 (100-1000 cells) were added to OP9, OP9-DL1 and/or OP9-DL4 feeder cells and incubated with differentiation medium supplemented with 10 ng/ml cytokines (IL-2, IL-23, IL-7 and IL-1β) (Tab. 9). Every 2-3 days the medium was changed, the supernatants were frozen at -20°C and 10 ng/ml IL-2, IL-23, IL-7, and IL-1β were added to the culture. After short- (3 days) or long-term (10-12 days) culture, the NKp44- ILC3s were analyzed or sorted and used for further experiments. The supernatants were analyzed with the Legendplex assay to detect cytokine secretion.

2.2.8   Generation of intestinal organoids

A piece of a biopsy was used to isolate intestinal crypts. The intestinal crypts containing stem cells were isolated, passaged, thawed and cryopreserved according to IntestiCult Organoid Growth Medium (OGM) protocol (STEMCELL™ TECHNOLOGIES Inc). More precisely, tissue samples were washed with ice-cold PBS and minced in small pieces. After centrifugation, tissue pieces were incubated 30 min in Gentle Cell Dissociation Reagent (GCDR, STEMCELL). After centrifugation, ice-cold DMEM + 1 % BSA was added and crypts were removed from tissue by vigorously pipetting. 1000 crypts/matrigel dome were plated in a 24 well plate. OGM with gentamycin (50 µg/mL) (Tab.10) was added and changed every 2-3 days. After 5-7 days, the organoids were splitted with GCDR, analyzed by immunofluorescence or quantitative real-time polymerase chain reaction (qRT-PCR).

2.2.9   Stimulation of organoids and Co-Culture

To stimulate organoids with recombinant cytokines (Tab. 9), organoids were cultured 5 days in OGM. After 5 days, organoids were stimulated with 10 ng/ml cytokines. After

20 h, the organoids were harvested using Cell Recovery solution (Corning^{TM}) for 30 min on ice. Organoids were pelleted and frozen at -80°C for RNA isolation or directly stained for immunofluorescence microscopy.

Additionally, organoids were co-cultured with duodenal NKp44$^-$ ILC3s according to previously published protocols (Schreurs *et al.*, 2021). Therefore, 45 cells/µl sorted NKp44$^-$ ILC3s (before expanded on OP9-DL4 cells for 12 days) were added to the organoids per Matrigel$^®$ dome and stimulated every two days with IL-1β and IL-23 to activate ILC3s. After 5 days, Organoids and ILC3s were harvested for RNA isolation, supernatants were collected and stored at -20°C until further use.

2.2.10 Flow cytometry analysis and cell sorting

To prepare the lymphocytes for flow cytometric analysis and sorting, the cells were thawed with thawing medium (Tab. 10), centrifuged at 350 g for 10 min and washed with DPBS. After centrifugation, dead cells were stained with Zombie Aqua^{TM} Fixable Viability Kit (Biolegend$^®$) for 10 min at room temperature (RT) in the dark, washed with DPBS and centrifuged. The cell surface was stained with specific antibodies (Tab. 5) for 10 min at RT in the dark. After further washing and centrifugation steps, the cells were analyzed or further prepared for cytokines or transcription factor analysis.

For intracellular analysis of transcription factors the eBioscience^{TM} Foxp3 Transcription Factor Staining Kit (Invitrogen^{TM}) and for cytokines the Cytofix/Cytoperm^{TM} Fixation/Permeabilization Kit (BD Bioscience) was used (Tab. 8). Permeabilization, fixation, and washing was performed according to manufacturer's protocols.

FACS data was acquired using a BD LSR-Fortessa^{TM} Cytometer (BD Bioscience) and analyzed using FlowJo$^®$ Software V10.7.1. Cell sorting was performed using a FACSAria^{TM} Fusion (BD Biosciences).

2.2.11 Legendplex

Cytokine secretion in supernatants was assessed by cytokine bead array (LEGENDplex™ Human Th Cytokine Panel Kit, Biolegend®), including data acquisition on a BD FACSCanto II and analysis using the software provided by the manufacturer (LEGENDplex™ Data Analysis Software).

2.2.12 Immunofluorescence microscopy

Intestinal tissues or organoids were fixed with 4 % paraformaldehyde (PFA) for 10 min at RT washed and incubated with blocking buffer for 30 min at RT (Tab. 10). After washing steps, tissue or organoids were incubated with primary antibodies in antibody buffer overnight at 4°C (Tab.6, Tab. 10). After washing, the secondary antibody was added for 45 min at RT in the dark (Tab. 6, Tab. 10). The nucleus was stained with 1:1000 dilution Hoechst for 1 min at RT. Trueblack®Lipofuscin (Biotium) was used to quench lipofuscin auto fluorescence for 5 min. After washing with water, the organoids or intestinal tissues were treated with one drop of Prolong™ Gold antifade reagent (Invitrogen™) to protect the fluorescent dyes from fading. A coverslip was then placed on top, and after drying, samples were imaged using a fluorescence microscope DM IL (Leica Microsystems GmbH).

2.2.13 RNA extraction

RNA was extracted from human intestinal biopsies and organoids. Therefore, fresh samples were frozen in NAP buffer and stored at -80°C until further use. RNA extraction was performed following the manufacturer's instructions (GeneJET RNA Purification Kit, Thermo FisherScientific). The lysis buffer was supplemented with 40 mM Dithiothreitol (DTT). The intestinal biopsies were homogenized by using a rotator-stator homogenizer (Bandelin electronic). The RNA concentration was measured with a NanoDrop™ 1000 (Thermo Fisher Scientific).

2.2.14 cDNA synthesis

The extracted RNA was transcribed into cDNA according to QuantiTect® Reverse Transcription Kit (Qiagen) in accordance with the manufacturer's protocol.

2.2.15 qRT-PCR

qRT-PCR was performed on a LightCycler® 96 Real-Time PCR System (Roche) with the Blue S' Green qPCR Kit (Biozym®) using the primer sets depicted in Tab.7 to measure relative mRNA expression. Relative target gene expression was calculated by the $2^{\wedge}\text{-}\Delta Cq$-method using mean expression of the house keeping gene *EEF1A1* as reference for normalization.

2.2.16 Bulk-RNA-Sequencing Analysis

For bulk-RNA-seq analysis, the normal (n=4) and adenomatous (n=4) mucosa of FAP patient and normal (n=4) mucosa of non-FAP patient was used and RNA was isolated according to manufacturer's protocol (GeneJET RNA purification kit, Thermo Scientific, Germany). Further analysis was performed from Deutsches Zentrum für Neurodegenerative Erkrankungen (DZNE, Bonn). The quality and quantity of the extracted RNA were assessed using the Agilent TapeStation 4200 and Nanodrop spectrophotometer, respectively. The extracted RNA was subjected to library preparation. The resulting libraries were sequenced. The raw sequencing data were subjected to quality control using FastQC, and then aligned to the reference human genome (GRCh38, ver. 33) using the STAR 2.7.3a for alignment. The aligned reads were quantified using the featureCounts program to generate raw read counts for each gene. The raw counts were normalized using the trimmed mean of M-values method, and differential gene expression analysis was performed using the DESeq2 (ver.1.38.1) package in R (ver. 4.2.2). The differentially expressed genes were defined as those with a fold change of > |1.5| and a false discovery rate (FDR) (or even p-value adjusted p-adj by BH) of <0.05.

2.2.17 Statistical Methods

Graphpad Prism software was used to analyze the data. To evaluate significance a paired or unpaired t-test with a two-tailed p value $p < 0.05$ and a 95 % confidence interval was used (Wilcoxon or Mann-Whitney test). For more than two datasets a one-way analysis of variance (ANOVA) was performed (Kruskal-Wallis (KW) test or Friedman test) and corrected for multiple comparisons with the False Discovery Rate (FDR). Significance was defined as $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***); $p < 0.0001$ (****) and $p > 0.05$ (ns). Correlations between two variables were tested by Pearson-test. Flow cytometric analyses with mean fluorescent intensity (MFI) was calculated using the geometric mean. Graphs show the mean ± standard deviation (SD). The figures were partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license.

3. Results

Duodenal adenoma formation in FAP patients, particularly the relationship between genotype and phenotype, is still poorly understood suggesting additional factors to contribute to the onset of the disease. Therefore, the goal of this study was to improve the understanding of the impact of duodenal lymphocytes in the duodenal adenoma formation of FAP patients.

Due to the scarcity of information on human ILCs, the innate counterpart of Th cells, in the GI tract and GI malignancies and their important role in cancer progression, a phenotypic and functional characterization of ILCs in duodenal adenomas of FAP patients, normal mucosa of FAP patients and normal mucosa of non-FAP controls was carried out (Fig. 6). To this end, tissue-resident lymphocytes were examined using multicolor flow cytometry, a qRT-PCR analysis of duodenal biopsies and bulk-RNA-seq was conducted. To investigate the effect of immune cells, duodenal organoids were generated.

Duodenal biopsy samples were collected from 88 FAP and 35 non-FAP patients. The adenomatous tissue of 22 FAP patients could be included in this study. From 2 FAP patients only adenomatous material could be obtained. Normal mucosa of non-FAP patients was used as control.
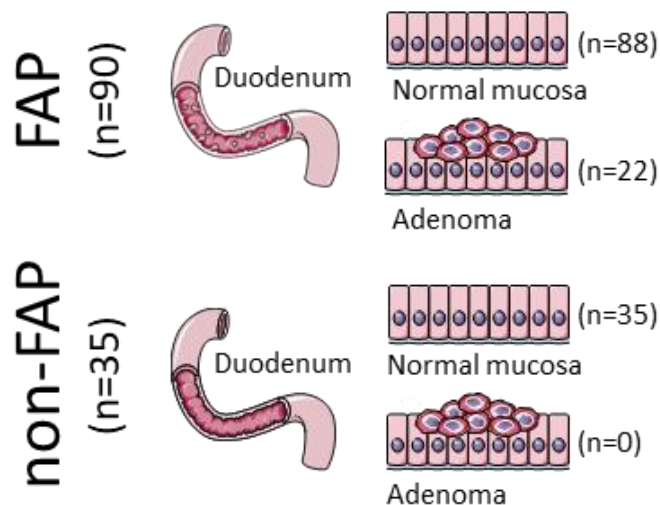
Fig. 6: Study design. Illustration of the patient's cohort and the number of samples in the duodenum.

3.1 Identification of increased ILCs in the duodenal normal mucosa and adenomas of FAP patients

First, we analyzed frequency and composition of the lymphocyte compartment in macroscopically normal and adenomatous duodenal mucosa of FAP patients compared to normal mucosa of non-FAP controls. To this end, tissue-resident lymphocytes were isolated, analyzed and gated according to previously published gating strategies and surface marker profiles (Freeman *et al.*, 2013). Various immune cells could be identified within the human duodenum (Fig. 7). Total lymphocytes were identified using only viable single cells with the marker CD45. T cells were gated using CD3 and further subdivided in cytotoxic T and Th cells with CD8 and CD4. Additionally, double positive CD4 and CD8 T cells were found. NKT cells expressed CD3 and CD56 and NK cells were CD3$^-$ CD56$^+$. B cells were identified using CD19.
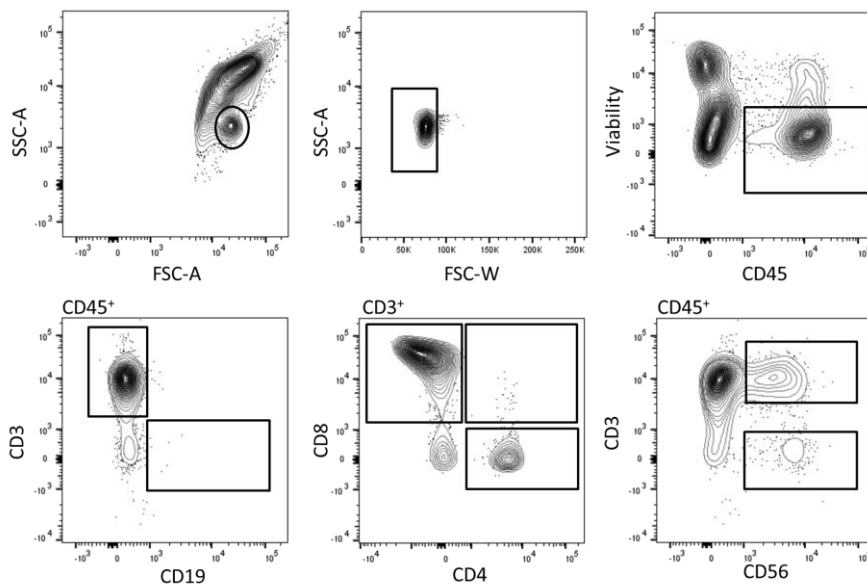
Fig. 7: Identification of lymphocytes in the duodenum. Gating strategy for lymphocytes defining T cells as CD45$^+$CD3$^+$CD4$^+$, CD8$^+$ or CD4$^+$CD8$^+$, B cells as CD45$^+$CD19$^+$, NKT cells as CD45$^+$CD56$^+$CD3$^+$, NK cells as CD45$^+$CD56$^+$CD3$^-$.

The frequency of total CD45$^+$ lymphocytes did not differ significantly between adenomatous (n=15) and normal mucosa (n=76) of FAP patients and normal mucosa (n=24) of non-FAP controls (Fig. 8A). There were no significant differences between the normal (n=7) and adenomatous (n=7) mucosa of FAP patients and the normal mucosa

(n=8) of non-FAP controls for any of the T cell subsets, including CD8$^+$, CD4$^+$ and CD4$^+$CD8$^+$ T cells, nor for B, NKT, and NK cells of CD45$^+$ cells (Fig. 8B-H). Nevertheless, we found a slight reduction of CD3$^+$, CD8$^+$ and CD4$^+$CD8$^+$ cells in the normal and adenomatous mucosa of FAP compared to non-FAP patients (Fig. 8B/C/E).
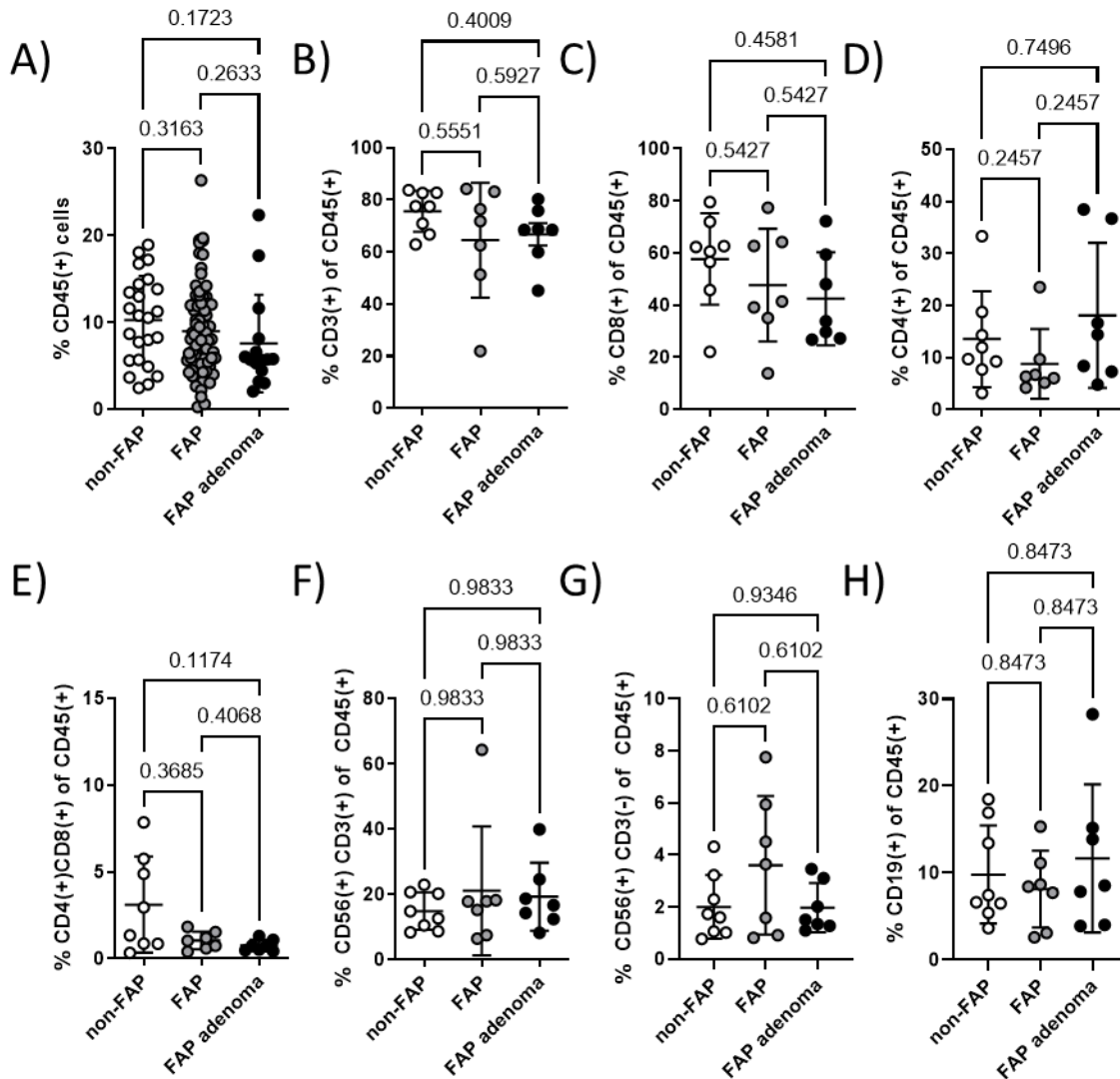
Fig. 8: Frequency of lymphocytes in the duodenal normal and adenomatous mucosa of FAP patients compared to normal mucosa of non-FAP controls. (A) Frequency of CD45$^+$ cells as percentages of the total cell count in the duodenal normal (n=76) and adenomatous (n=15) mucosa of FAP patients compared to normal mucosa (n=24) of non-FAP controls (B-H) Frequency of CD3$^+$ T cells, CD8$^+$ T, CD4$^+$ T, CD8$^+$CD4$^+$ T, CD56$^+$CD3$^+$ NKT, CD56$^+$CD3$^-$ NK and B cells as percentages of CD45$^+$ cells in the duodenal normal (n=7) and adenomatous (n=7) mucosa of FAP patients compared to normal mucosa (n=8) of non-FAP controls. Error bars showing SD. Statistical significance analyzed by Kruskal-Wallis (KW) test, corrected for multiple comparisons by controlling the FDR (Benjamini, Krieger and Yekutieli).

However, analyzing innate lymphoid cells (ILCs), defined as CD45$^+$ CD127$^+$ Lin$^-$ (CD3, CD4, CD5, CD14, CD19, CD20, TCRγδ, TCRαβ, BDCA-2, CD1a, CD34, NKp80, CD94, FcεR1a and CD123) (Bernink *et al.*, 2013; Björklund *et al.*, 2016) (Fig. 9A), we observed significantly increased frequencies of total duodenal ILCs (CD45$^+$ Lin$^-$ CD127$^+$) in FAP patients (n=76) with highest numbers found in adenomatous (n=15) tissue (Fig. 9B). Given these findings and the known roles of ILCs in malignancies (Yuan *et al.*, 2021), we performed a more in-depth analysis of this particular lymphocyte subset.
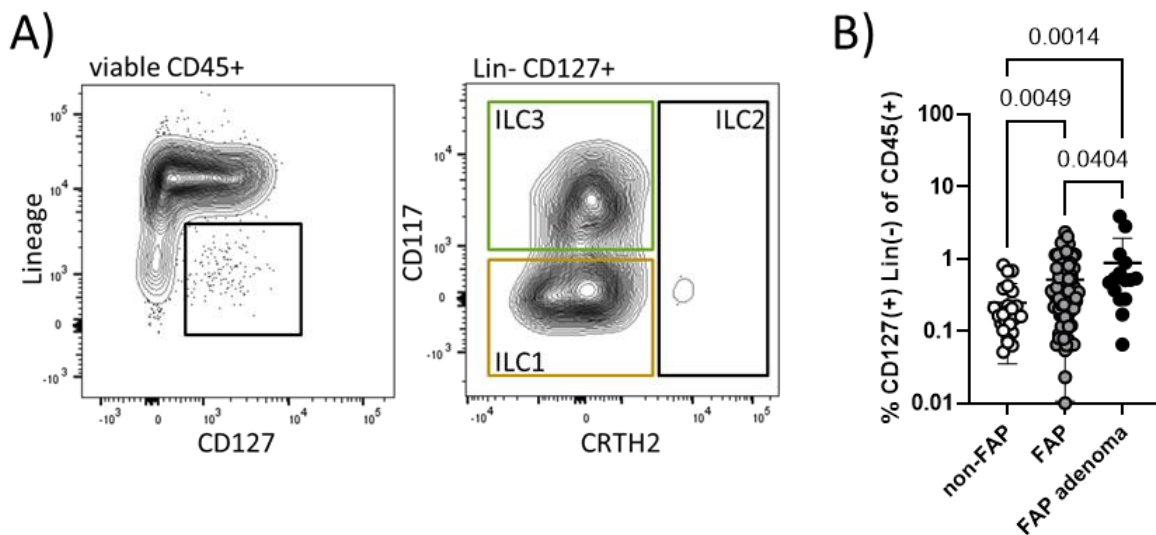
Fig. 9: Identification of increased ILCs in the duodenum of FAP patients. (A) ILCs were gated as single viable CD45$^+$ Lin$^-$ (CD3, CD4, CD5, CD14, CD19, CD20, TCRγδ, TCRαβ, BDCA-2, CD1a, CD34, NKp80, CD94, FcεR1a, CD123) CD127$^+$ cells. ILC subsets are divided with CD117 and CRTH2. (B) Frequency of total ILCs (Lin$^-$ CD127$^+$) as percentages of viable CD45$^+$ cells in normal (n=76) and adenomatous (n=15) mucosa of FAP patients and normal mucosa (n=24) of non-FAP controls. Error bars showing SD. Statistical significance analyzed by KW test, corrected for multiple comparisons by controlling the FDR (Benjamini, Krieger and Yekutieli).

3.2 Phenotypical analysis of duodenal ILCs

Following standard gating strategies frequencies of CD117$^-$ CRTH2$^-$ ILC1s and CD117$^+$ CRTH2$^-$ ILC3s could be detected in duodenal tissue, whereas only negligible numbers of CRTH2$^+$ ILC2s were found, confirming previous reports of our group (Krämer *et al.*, 2017) (Fig. 9A, Fig. 11).

To validate the identity of these ILC subsets, ILC1s and ILC3s were further characterized regarding expression of surface markers and transcription factors (TF) (Artis and Spits, 2015; Simoni and Newell, 2018).

ILC3s expressed the transcription factor RORγt but were negative for GATA3 a TF characteristic for ILC2, the ILC1-associated TF T-bet and EOMES, a NK cell specific TF (Fig. 10A/B) (Artis and Spits, 2015; Simoni and Newell, 2018). ILC3s also expressed other ILC3-specific surface markers such as IL1R1, CD56, CD103, CD200R and NKp44 (Fig. 10C). The ILC1-specific transcription factor T-bet was hard to be visualize in any subset (Fig. 10B). However, ILC1s expressed specific surface markers such as CD56, CD49a and NKp44 (Fig 10C), which altogether confirmed the identity of this ILC subpopulation.
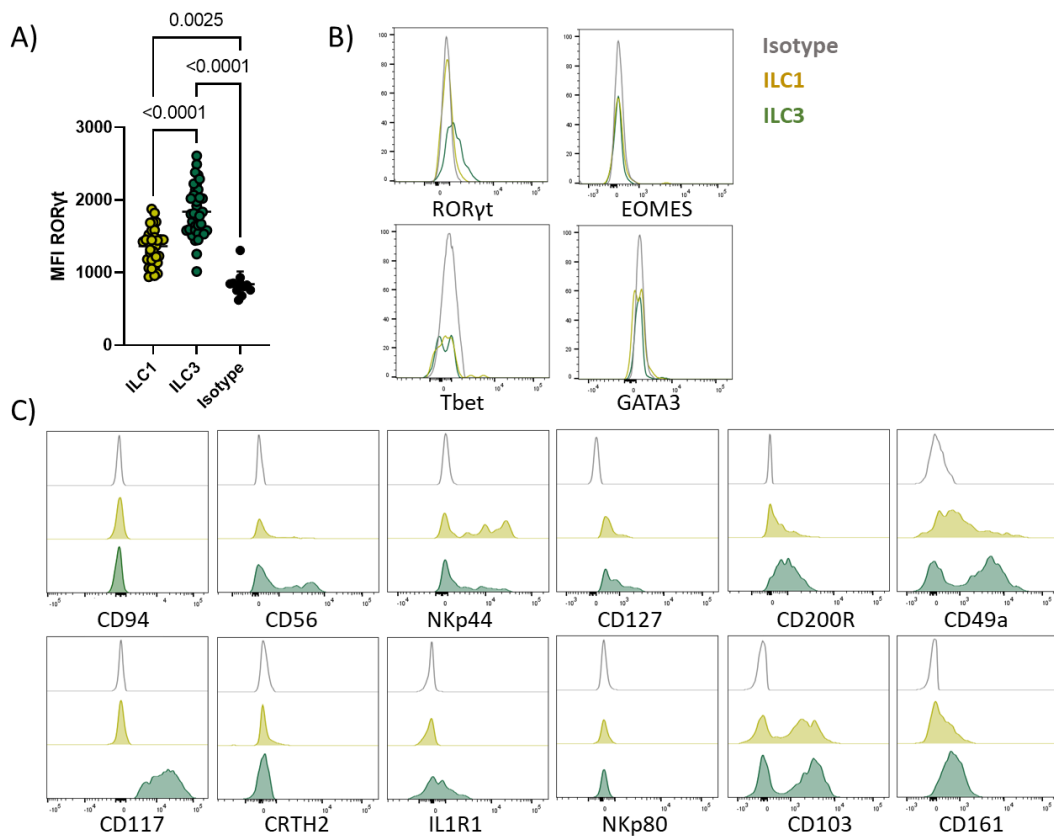
Fig. 10: Phenotype of ILC1 and ILC3 in the duodenum. (A) Mean fluorescent intensity (MFI) of RORγt in ILC1 (yellow, n=31), ILC3 (green, n=36) and an isotype control (grey, n=11). (B) Representative histogram showing expression of the transcription factors RORγt, EOMES, T-bet and GATA3 in ILC1, ILC3 and an isotype control. (C) Representative histograms displaying ILC-specific surface markers of ILC1 (CD117⁻CRTH2⁻), ILC3 (CD117⁺CRTH2⁻) and an isotype control. Error bars showing SD. Statistical significance analyzed by KW test, corrected for multiple comparisons by controlling the FDR (Benjamini, Krieger and Yekutieli).

Comparing the frequencies of ILC subsets in the normal mucosa (n=76) and adenomas (n=15) of FAP patients as well as in normal non-FAP (n=24) mucosa, we found numbers of ILC1s to be significantly increased in the normal and adenomatous mucosa of FAP patients (Fig. 11A).

The most prominent alterations, however, were found for the ILC3 subset. Here, we not only observed significantly increased numbers in normal and adenomatous FAP mucosa compared to controls but also significant differences between non-adenoma and adenoma tissue in FAP patients (Fig. 11A/B). Accordingly, ILC3s represented the dominant ILC subset in FAP adenomas, suggesting these cells to play a prominent role in FAP-associated adenoma formation (Fig.11B).
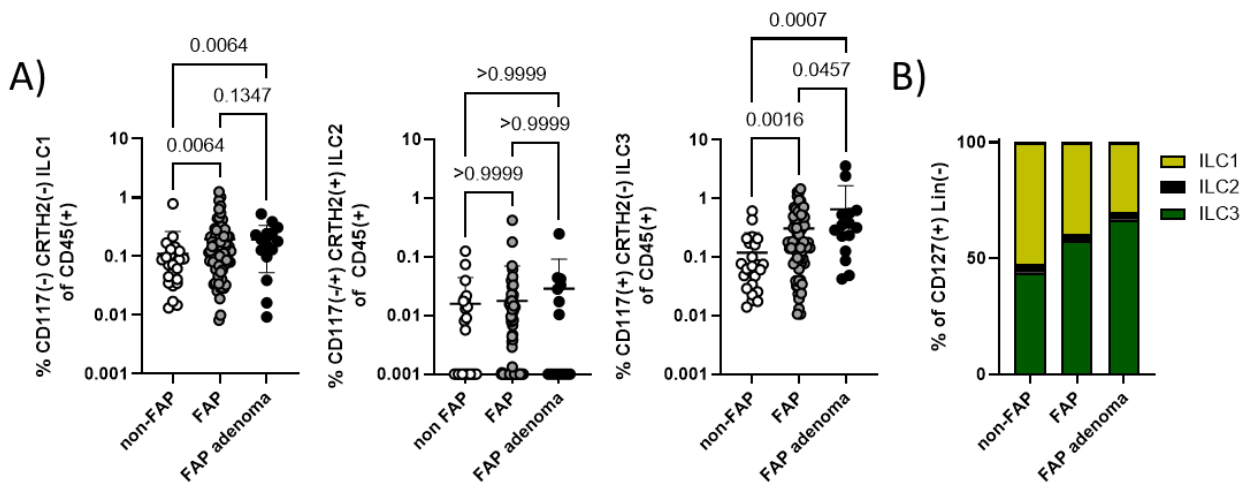
Fig. 11: Increased ILC3 in normal and adenomatous mucosa of FAP patients. (A) Frequency of CD117-CRTH2- ILC1, CRTH2+ ILC2 and CD117+CRTH2- ILC3 among viable CD45 in non-FAP (n=24) controls and normal (n=76) and adenomatous (n=15) mucosa of FAP patients. (B) Mean frequency of CD117-CRTH2- ILC1 (yellow), CRTH2+ ILC2 (black) and CD117+CRTH2- ILC3 (green) of CD127+ total ILCs. Error bars showing SD. Statistical significance analyzed by KW test, corrected for multiple comparisons by controlling the FDR (Benjamini, Krieger and Yekutieli).

Further analyses of ILC3 subsets demonstrated that frequencies of NKp44- ILC3s were significantly whereas NKp44+ ILC3s were only slightly increased in normal and adenomatous mucosa of FAP patients (Fig. 12A/B). Additionally, the FAP-associated duodenal accumulation of ILC3s were mainly attributable to an increase in NKp44- ILC3s

within total ILCs (Fig.12C). The frequency of NKp44$^-$ ILC3s was already increased in the normal mucosa of FAP patients with SS 0 without any adenomas (Fig. 12D).
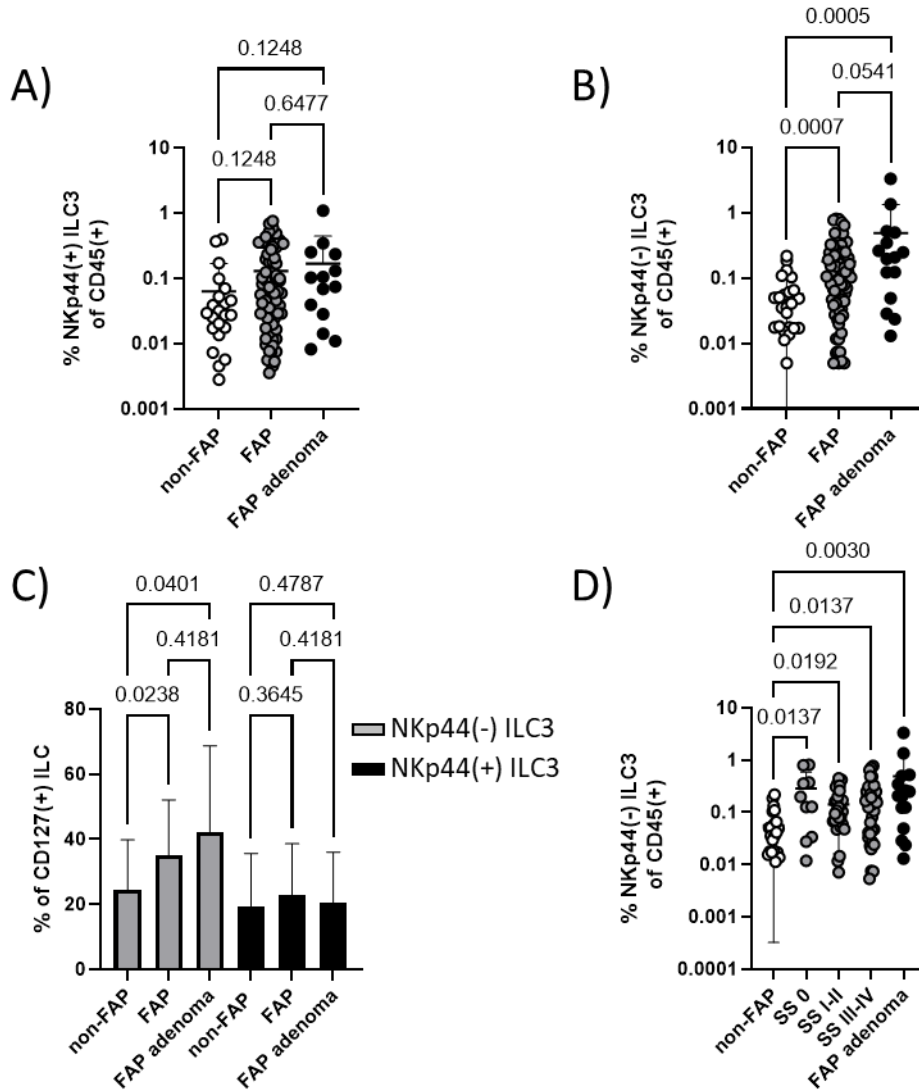
Fig. 12: Increased frequencies of NKp44$^-$ ILC3s in the normal and adenomatous mucosa of FAP patients. (A-B) Frequency of NKp44$^+$ (A) and NKp44$^-$ (B) CD117$^+$CRTH2$^-$ ILC3 among viable CD45$^+$ cells in the normal mucosa of non-FAP (n=24) controls and normal (n=76) and adenomatous (n=15) mucosa of FAP patients. (C) Frequency of NKp44$^+$ and NKp44$^-$ ILC3 among CD127$^+$ ILCs in the normal mucosa of non-FAP (n=20) controls and normal (n=62) and adenomatous (n=12) mucosa of FAP patients. (D) Frequency of NKp44$^-$ ILC3 among CD45$^+$ cells in the normal mucosa of non-FAP (n=24) controls and normal (Spigelman Stage (SS) 0: n=10, SS I-II: n=28, SS III-IV: n=38) and adenomatous (n=15) mucosa of FAP patients. Error bars showing SD. Statistical significance analyzed by KW test, corrected for multiple comparisons by controlling the FDR (Benjamini, Krieger and Yekutieli).

Moreover, phenotypic characterization of duodenal ILC3s did not reveal any significant differences in the expression of cell surface markers or transcription factors between non-FAP controls and normal and adenomatous mucosa of FAP patients (Fig.13).
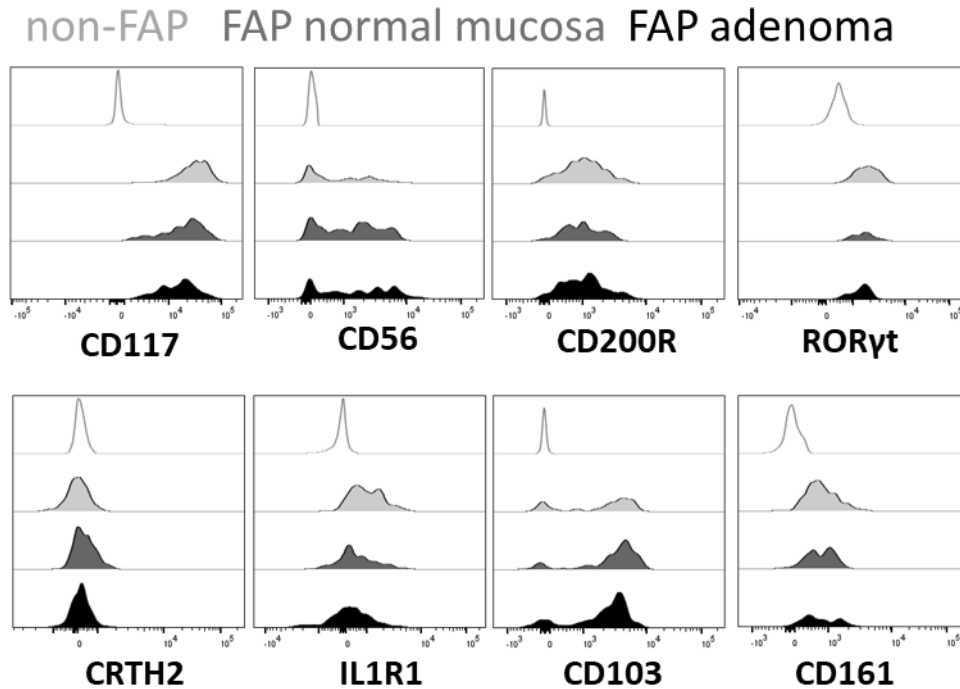
Fig. 13: ILC3-specific markers in the duodenum of FAP and non-FAP patients. Representative histograms displaying ILC-specific markers of duodenal ILC3s in the normal mucosa of non-FAP, FAP and in FAP adenoma with isotype control (upper lane).

3.3 Functional analysis of ILC3s in FAP and non-FAP patients

According to our finding of increased ILC3s in FAP duodenal tissue and based on the knowledge that ILC3s have an importance in the intestinal homeostasis due to their cytokine secretion ability, we also analyzed the overall functional potential of duodenal ILC3s in non-FAP controls, normal and adenomatous tissue of FAP patients.

Thus, we stimulated tissue-resident lymphocytes with PMA (50 ng/ml) and Ionomycin (1 µg/ml) (P/I) for 4 h in the presence of BFA for the last 3 h to stain intracellularly for ILC-specific cytokines and analyzed them by flow cytometry (Fig. 14).

ILC3s produced the cytokines IL-17A, TNF-α, IL-2 and IL-8 whereas IL-22 and IFN-γ could be barely observed after P/I stimulation (Fig. 14, Fig. 15C).
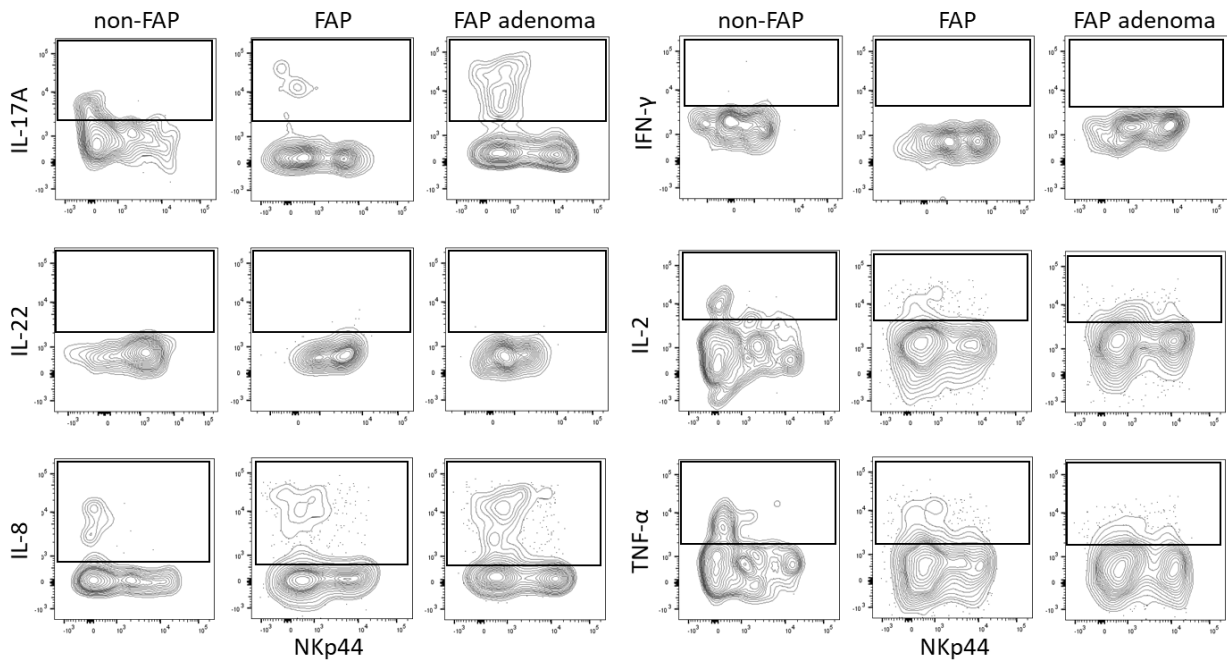
Fig. 14: Produced ILC3 cytokines. Representative gating of intracellular cytokine (IL-17A, IL-22, IL-8, IFN-γ, IL-2 and TNF-α) production of ILC3s in duodenal adenomatous and normal mucosa of FAP and non-FAP patients.

Comparing ILC3s of normal mucosa of non-FAP (n≥8) controls, the normal mucosa (n≥27) and adenomas (n≥4) of FAP patients in terms of their cytokine production, only frequency of IL-17A-producing ILC3s differed significantly (Fig. 15A). Other cytokines such as IL-22, IL-8, IL-2, TNF-α and IFN-γ did not show significant differences between the cohorts (Fig. 15A/C). Increased IL-17A production of ILC3s was already seen in normal duodenal tissue of FAP patients but was even higher in FAP adenomas (Fig. 15A).

FAP-associated increase in IL-17A production by ILC3s was also confirmed when the proportion of IL-17A$^+$ ILC3s relative to the total CD45$^+$ lymphocyte population was analyzed (Fig. 15B).
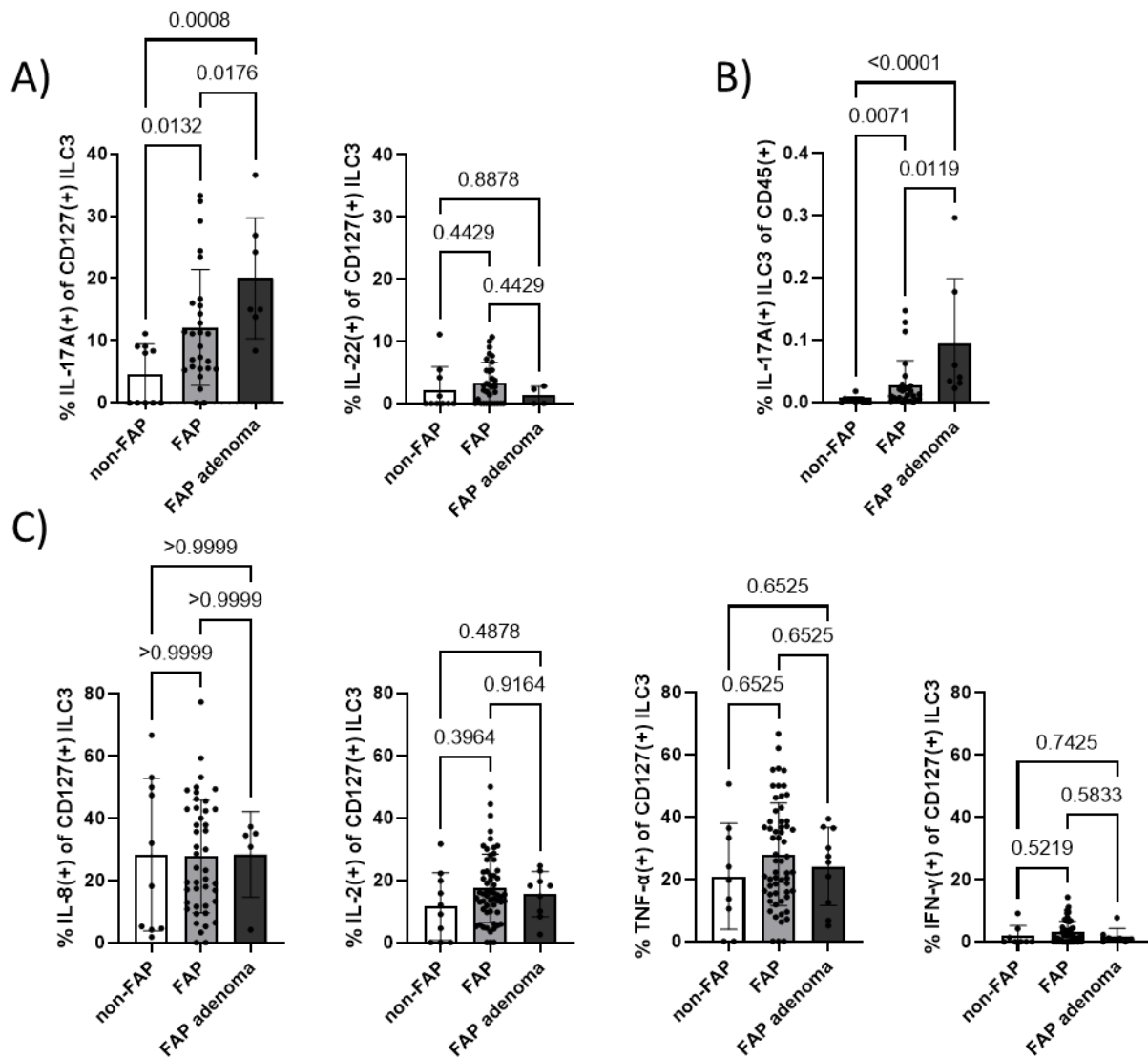
Fig. 15: Increased IL-17A production of ILC3s in FAP patients. (A) Intracellular IL-17A and IL-22 production of ILC3s in adenomatous (n≥4) and normal mucosa (n≥27) of FAP and non-FAP (n=10) patients. (B) Frequency of IL-17A⁺ ILC3 among CD45⁺ cells in duodenal adenomatous (n=7) and normal mucosa (n=27) of FAP and non-FAP (n=10) patients. (C) Intracellular IL-8, IL-2, TNF-α and IFN-γ percentages of ILC3s in adenomatous (n≥5) and normal mucosa (n≥44) of FAP and non-FAP (n≥8) patients. Error bars showing SD. Statistical significance analyzed by KW test, corrected for multiple comparisons by controlling the FDR (Benjamini, Krieger and Yekutieli).

To see whether there is a difference in IL-17A production between NKp44⁻ and NKp44⁺ ILC3s, we next stratified ILC3s according to NKp44 expression, which confirmed NKp44⁻ ILC3s as the main producers of IL-17A (Fig. 16A).

Comparing the cohorts regarding IL-17A production of ILC3 subsets, the significantly increased IL-17A production of ILC3s in the normal mucosa (n=26) and adenomas (n=7) of FAP patients could only be observed in NKp44⁻ ILC3s and not in NKp44⁺ ILC3s (Fig. 16B).
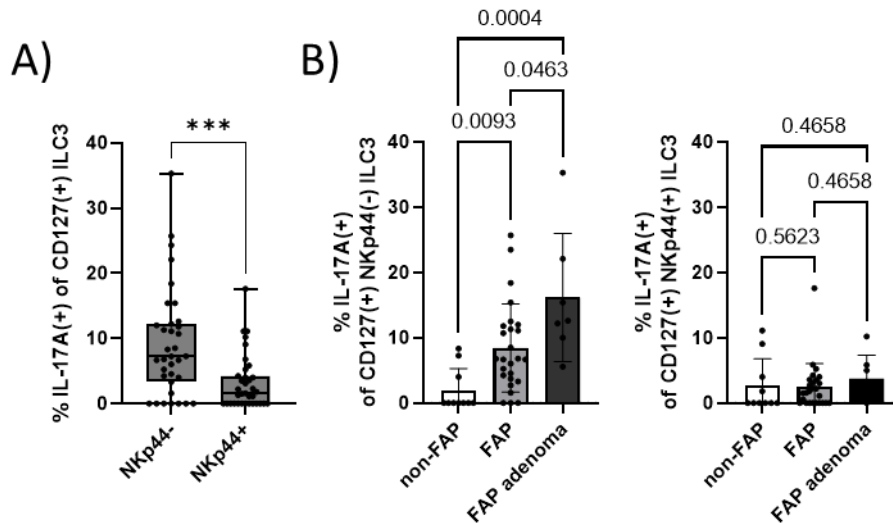
Fig. 16: Elevated IL-17A-producing NKp44⁻ ILC3 in FAP patients. (A) Percentages of IL-17A⁺ cells of NKp44⁺ and NKp44⁻ ILC3s (n=35) in the duodenum. (B) Percentages of IL-17A⁺ cells of NKp44⁺ and NKp44⁻ ILC3s in duodenal adenomatous (n=7) and normal mucosa (n=26) of FAP and non-FAP patients (n=10). Error bars showing SD. Statistical significance analyzed by Mann-Whitney test (A) and KW test (B), corrected for multiple comparisons by controlling the FDR (Benjamini, Krieger and Yekutieli).

3.4 Increase in IL-17A-producing NKp44⁻ ILC3s in FAP is a specific finding in the duodenum

To ascertain whether the aforementioned findings are also seen in other compartments, we also examined colon tissue and PBMCs of FAP patients in addition to the duodenum. Therefore, blood of non-FAP (n=15) and FAP (n=37) patients as well as colonic biopsies of non-FAP (n=11) and FAP (n=23) patients were collected. Additionally, 12 colonic adenomas of FAP patients were analyzed.

As could be expected, frequencies and composition of the ILC compartment differed significantly between duodenum, the colon tissue and peripheral blood with highest numbers of total CD127⁺ ILCs and CD117⁺ CRTH2⁻ ILC3s found in colon samples (Fig.

17A/C, Fig. 9, Fig. 11). Frequencies of total CD127$^+$ ILCs and CD117$^+$CRTH2$^-$ ILCs, which are considered to represent circulating ILC precursor (ILCPs) rather than mature ILC3 (Lim *et al.*, 2017) were lowest in PBMCs and did not differ between the groups (Fig. 17B/C). In contrast to our findings in the duodenum, we observed a marked reduction in both total ILCs and ILC3s in FAP adenoma compared to normal FAP mucosa (Fig. 17A) but an increase in ILC1s (Fig. 17C).
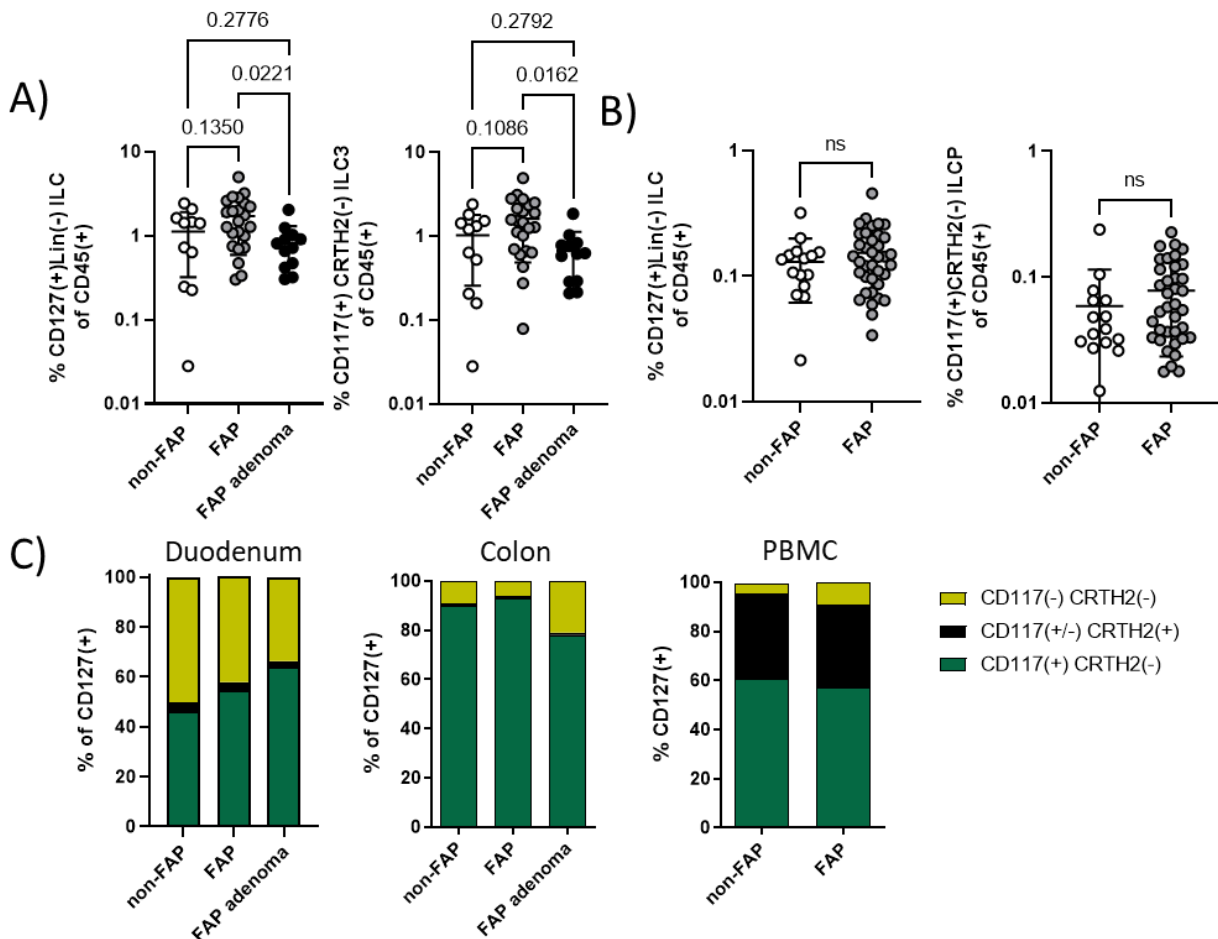
Fig. 17: Increased ILC3s in FAP adenoma is duodenum specific. (A) Frequency of total Lin$^-$ CD127$^+$ ILCs and CD117$^+$ CRTH2$^-$ ILC3s among CD45$^+$ cells in adenomatous (n=12) and normal (n=23) mucosa of FAP and non-FAP (n=11) patients in the colon. (B) Frequency of total Lin$^-$ CD127$^+$ ILCs and CD117$^+$ CRTH2$^-$ ILCPs among CD45$^+$ cells in peripheral blood of FAP (n=37) and non-FAP (n=15) patients (C) ILC distribution in adenomatous and normal mucosa of FAP and non-FAP patients in the duodenum and colon, and peripheral blood of both cohorts. Error bars showing SD. Statistical significance analyzed by Mann-Whitney test (B) and KW test (A), corrected for multiple comparisons by controlling the FDR (Benjamini, Krieger and Yekutieli).

Further analysis confirmed that ILC3s in the colon are mainly composed of cells expressing the surface marker NKp44 (Fig. 18A) and showed that the decreased frequency of total ILC3s in colon adenomas was primarily caused by a decrease in NKp44$^+$ ILC3s (Fig. 18B). PBMCs were only composed of NKp44$^-$ ILCPs (Fig. 18A).
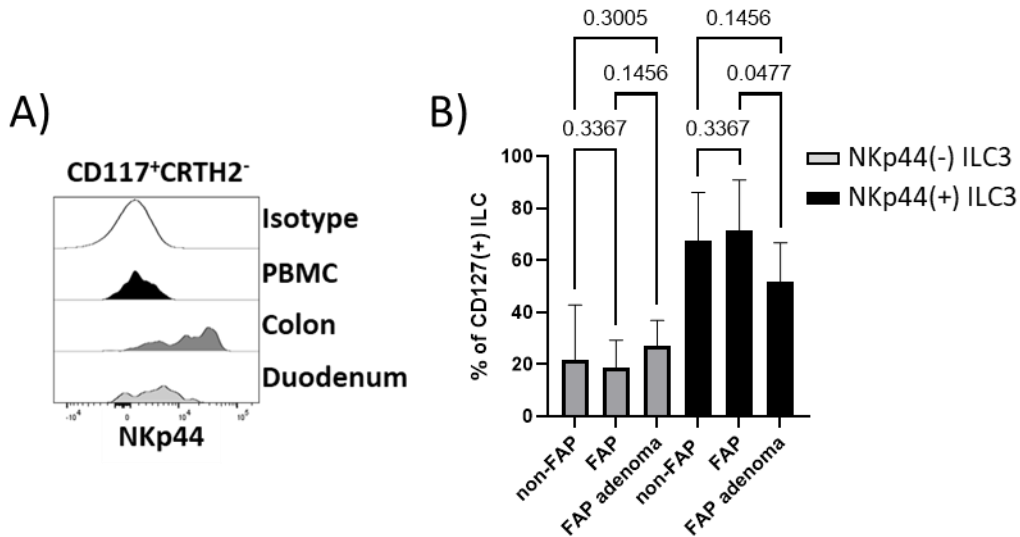
Fig. 18: Differential expression of NKp44 in colonic and duodenal ILC3. (A) Representative histogram of the NKp44 expression in CD117$^+$CRTH2$^-$ ILCs in the duodenum, colon and peripheral blood with isotype control. (B) Frequency of NKp44$^+$ and NKp44$^-$ ILC3s among CD127$^+$ ILCs in adenomatous (n=12) and normal mucosa (n=23) of FAP and non-FAP patients (n=11) in the colon. Error bars showing SD. Statistical significance analyzed by KW test corrected for multiple comparisons by controlling the FDR (Benjamini, Krieger and Yekutieli).

Next, we compared the IL-17A production of colonic and duodenal ILC3s. To this end, tissue-resident colonic lymphocytes were stimulated with P/I and tested for their capacity to produce IL-17A.

In contrast to our findings in the duodenum, functional analysis of colonic ILC3s did not reveal any significant changes in the IL-17A production between the normal and adenomatous mucosa of FAP patients and normal mucosa of non-FAP controls (Fig. 19A). The IL-17A production of ILC3s was significantly higher in the duodenum than in the colon in the normal and adenomatous mucosa of FAP patients (Fig.19A/B). These differences were only found in FAP and not in non-FAP patients.
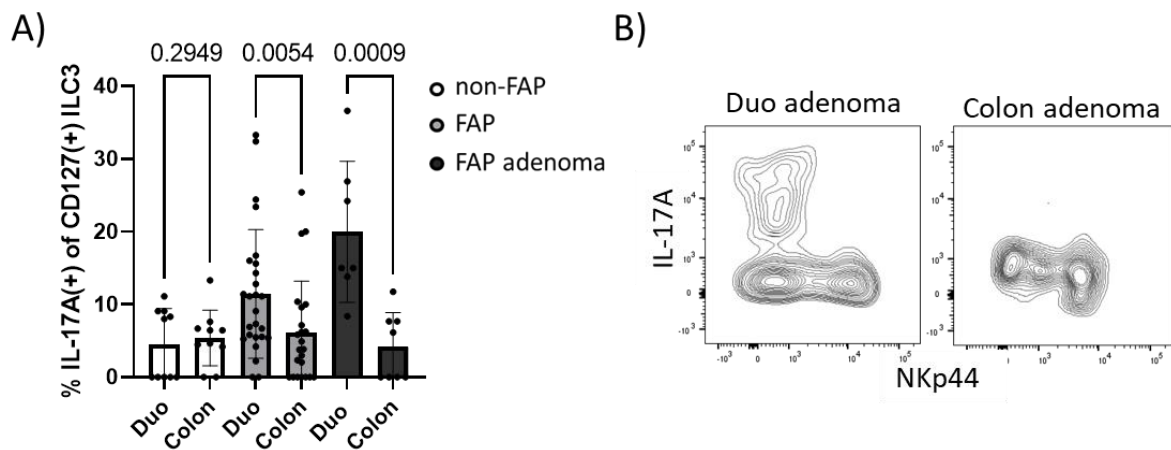
Fig. 19: Increased IL-17A-producing ILC3s in FAP patients is duodenum specific.
(A) Percentages of IL-17A⁺ ILC3s in colonic and duodenal adenomatous (n≥7) and normal mucosa (n≥23) of FAP and non-FAP patients (n=10). (B) Representative intracellular IL-17A production of ILC3s in adenomatous mucosa of FAP patients in the duodenum and colon tissue. Error bars showing SD. Statistical significance analyzed by KW test corrected for multiple comparisons by controlling the FDR (Benjamini, Krieger and Yekutieli).

Similar observations were made when colon ILC3s were stratified according to NKp44 expression as neither NKp44⁺ nor NKp44⁻ ILC3s displayed any significant alteration in IL-17A production in FAP compared to non-FAP controls (Fig. 20A/B). In summary, the increase of IL-17A⁺ NKp44⁻ ILC3s is a duodenum-specific effect.
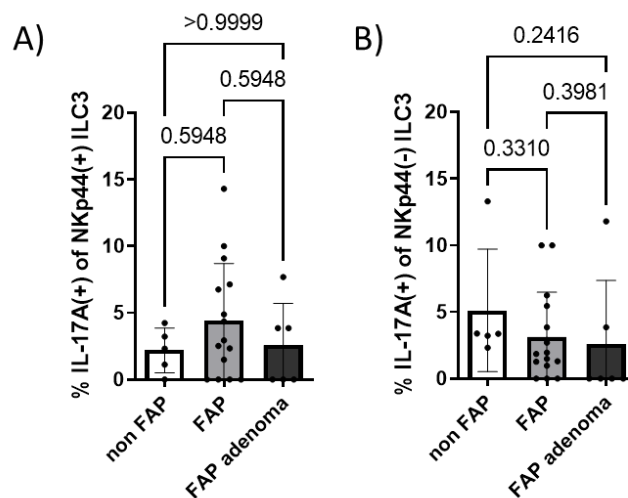
Fig. 20: IL-17A production of colonic NKp44⁺ and NKp44⁻ ILC3s. (A-B) Percentages of IL-17A production of NKp44⁺ (A) and NKp44⁻ (B) ILC3s in colonic adenomatous (n=6) and normal (n=15) mucosa of FAP and non-FAP (n=5) patients. Error bars showing SD. Statistical significance analyzed by KW test, corrected for multiple comparisons by controlling the FDR (Benjamini, Krieger and Yekutieli).

3.5 Duodenal microenvironment

3.5.1 Increased ILC3-related factors in FAP tissue

ILC biology is regulated by the local microenvironment (Vivier *et al.*, 2018). Particularly, it has been demonstrated that cytokines such as IL-1β and IL-23A and Notch ligands Delta-like (DL)1 and DL4 are crucial for controlling intestinal ILC development, maturation, and function (Heinrich *et al.*, 2022; Golub, 2021).

Thus, the mucosal ILC-specific cytokine microenvironment and Notch ligand expression was analyzed to better understand the factors involved in duodenal accumulation of IL-17A-producing ILC3s in FAP. Therefore, qRT-PCRs of normal (n=32) and adenomatous (n=8) tissues of FAP patients and normal tissues (n=13) of non-FAP patients were performed.

We found that the relative mRNA levels of a number of cytokines involved in the expansion, differentiation and activation of ILCs (*IL33*, *IL1B*, *IL23A*, *TGFb1* and *IL12*) (Vivier *et al.*, 2018) were significantly increased in the adenomatous mucosa of FAP patients compared to non-FAP control tissue (Fig. 21).

With regard to the observed FAP-associated duodenal accumulation of IL-17A-producing ILC3s, the finding of significantly increased mRNA levels of *IL23A* and *IL1B* was of particular interest, as these cytokines were also significantly increased in the normal mucosa of FAP patients and play an important role in the activation of cytokine secretion of ILC3s (Fig. 21).
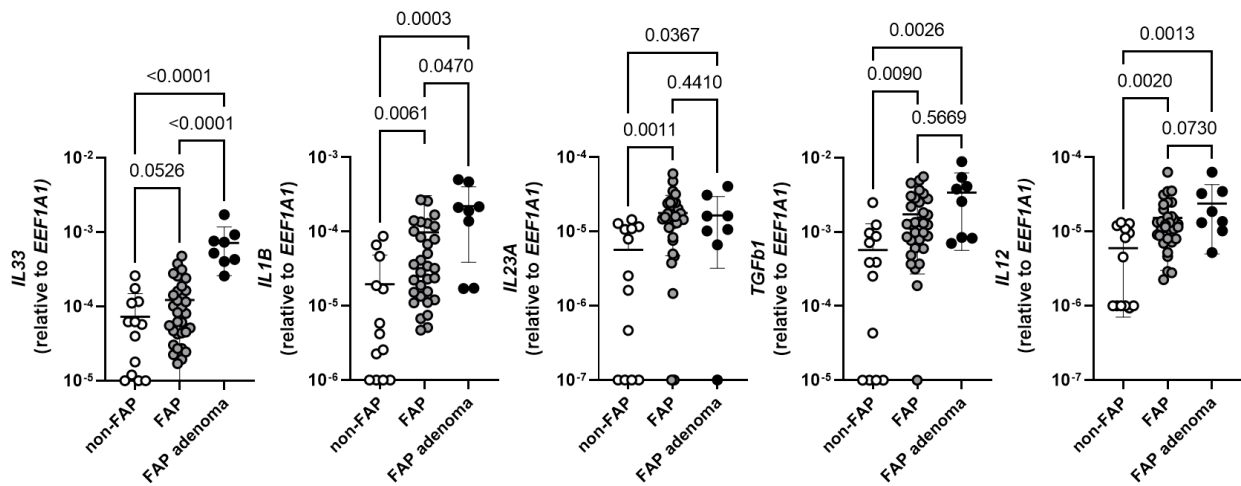
Fig. 21: Increased cytokines in the duodenum of FAP patients. *IL33, IL1B, IL23A, TGFb1* and *IL12* mRNA expression in duodenal normal (n=32) and adenomatous (n=8) mucosa of FAP and non-FAP (n=13) patients in relation to *EEF1A1*. Error bars showing SD. Statistical significance analyzed by KW test, corrected for multiple comparisons by controlling the FDR (Benjamini, Krieger and Yekutieli).

To test for a potential relation between the increased number of IL-17A-producing NKp44⁻ ILC3s in the FAP duodenum and the expression of *IL1B* or *IL23A*, we performed correlation analyses. These revealed a significant correlation between the number of IL-17A⁺ NKp44⁻ ILC3s and the mRNA expression levels of both cytokines (Fig. 22).
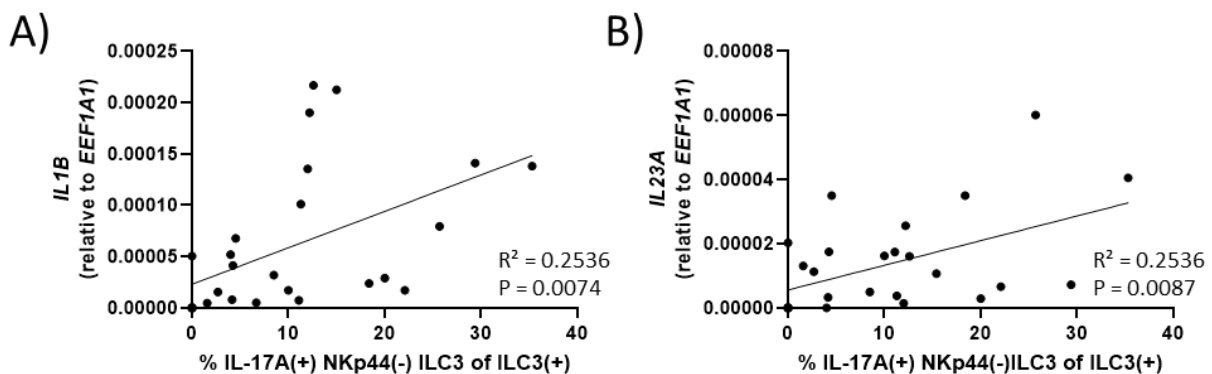
Fig. 22: Correlation analyses. (A-B) Correlation of duodenal mRNA levels of *IL1B* (A) and *IL23A* (B) with IL-17A⁺ NKp44⁻ ILC3s.

In addition to analyzing cytokine expression in duodenal tissue, we also performed qRT-PCR on normal (n=9) and adenomatous (n=8) colon tissue from both FAP patients and

patients without FAP (n=9). The results showed that, in contrast to duodenal tissue in the colon, the relative mRNA levels of *IL1B* were not significantly increased in either normal or adenomatous colon tissue from FAP patients (Fig. 23A). Regarding *IL23A*, a slightly increased relative mRNA expression was found in the colonic tissue of FAP patients compared with the normal mucosa of non-FAP controls. However, this difference was also not statistically significant (Fig. 23B), further highlighting the differences between FAP-associated alterations in colon and duodenum.
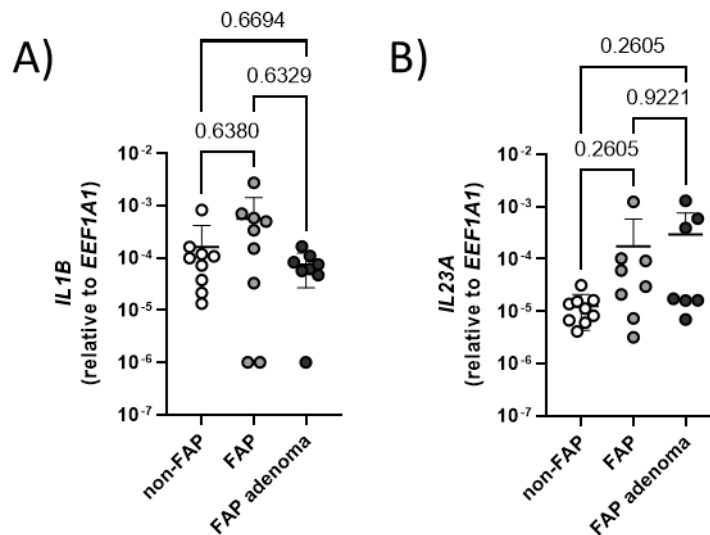
Fig. 23: Higher *IL1B* and *IL23A* levels are duodenum-specific in FAP. (A-B) *IL1B* (A) and *IL23A* (B) mRNA expression in colon adenomatous (n=8) and normal (n=9) mucosa of FAP and non-FAP (n=9) patients in relation to *EEF1A1*. Error bars showing SD. Statistical significance analyzed by KW test, corrected for multiple comparisons by controlling the FDR (Benjamini, Krieger and Yekutieli). Experiment performed in cooperation with Meike Wagner.

Next, we analyzed the relative mRNA expression of *DL1* and *DL4* in the duodenum, which was significantly increased in both adenomatous (n=8) and normal (n=32) duodenal mucosa of FAP patients compared with non-FAP (n=13) patients (Fig. 24A). Of note, we found frequency of duodenal IL-17A$^+$ NKp44$^-$ ILC3s to correlate significantly with mRNA levels of both *DL4* and *DL1* (Fig. 24C). No such differences were found in colon tissue (Fig. 24B).
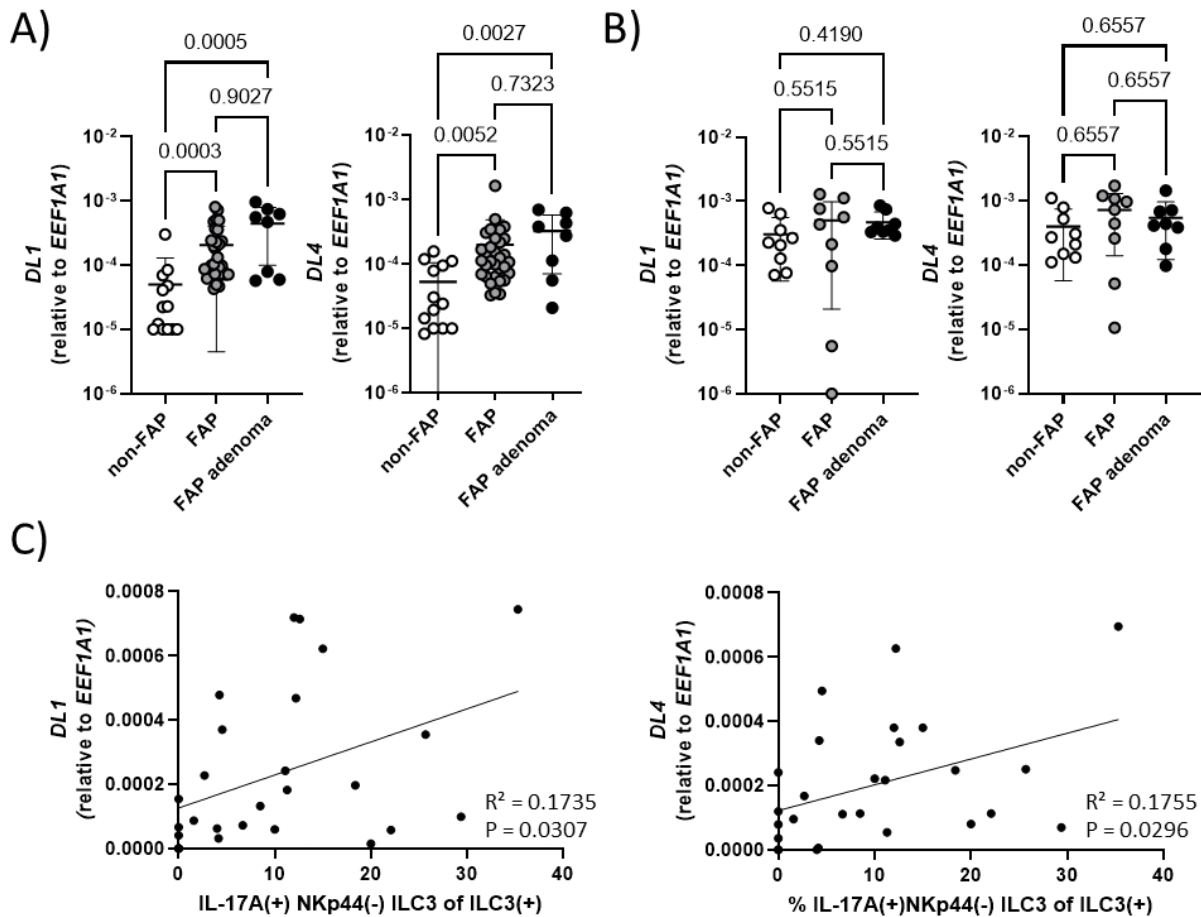
Fig. 24: Higher *DL1 and DL4* levels in the duodenum of FAP patients. (A-B) *DL1* and *DL4* mRNA expression in duodenal (A) and colonic (B) adenomatous (n=8) and normal (n≥9) mucosa of FAP and non-FAP (n≥9) patients in relation to *EEF1A1*. (C) Correlation of duodenal *DL1* and *DL4* to IL-17A⁺ NKp44⁻ ILC3s. Error bars showing SD. Statistical significance analyzed by KW test, corrected for multiple comparisons by controlling the FDR (Benjamini, Krieger and Yekutieli). Experiment performed in cooperation with Meike Wagner.

We then carried out *in-vitro* cultures to further support a role for elevated IL-1β/IL-23 and DL1/DL4 expression on ILC3 expansion and activity in FAP patients. After 12 days of culturing freshly sorted duodenal NKp44⁻ ILC3s (n=3) on DL4-expressing OP9 (OP9-DL4) cells in the presence of IL-1β, IL-23, IL-7 and IL-2, we observed an increased cell count of NKp44⁻ ILC3s and robust IL-17A concentrations in the resulting supernatants, indicating that ILC3s can be expanded and activated under these conditions (Fig. 25A/B). To better understand the role of DL1 vs DL4 stimulation, we next sorted NKp44⁻ ILC3s (n=3) on either OP9, OP9-DL1 or OP9-DL4 feeder cells. In this approach, tonsil ILC3s were used

instead of duodenal ILC3s because direct co-culture was not possible due to the relatively small amount of duodenal ILCs available and the fact that after *in-vitro* expansion the responsiveness of the cells to stimulation was significantly altered (Fig. 25C). Analyzing the resulting supernatants after three days of culture in the presence of IL-1β and IL-23 demonstrated highest IL-17A concentrations in the presence of OP9-DL4 feeder cells, indicating an important role of DL4 in regulation of NKp44⁻ ILC3 activity (Fig. 25C/D).
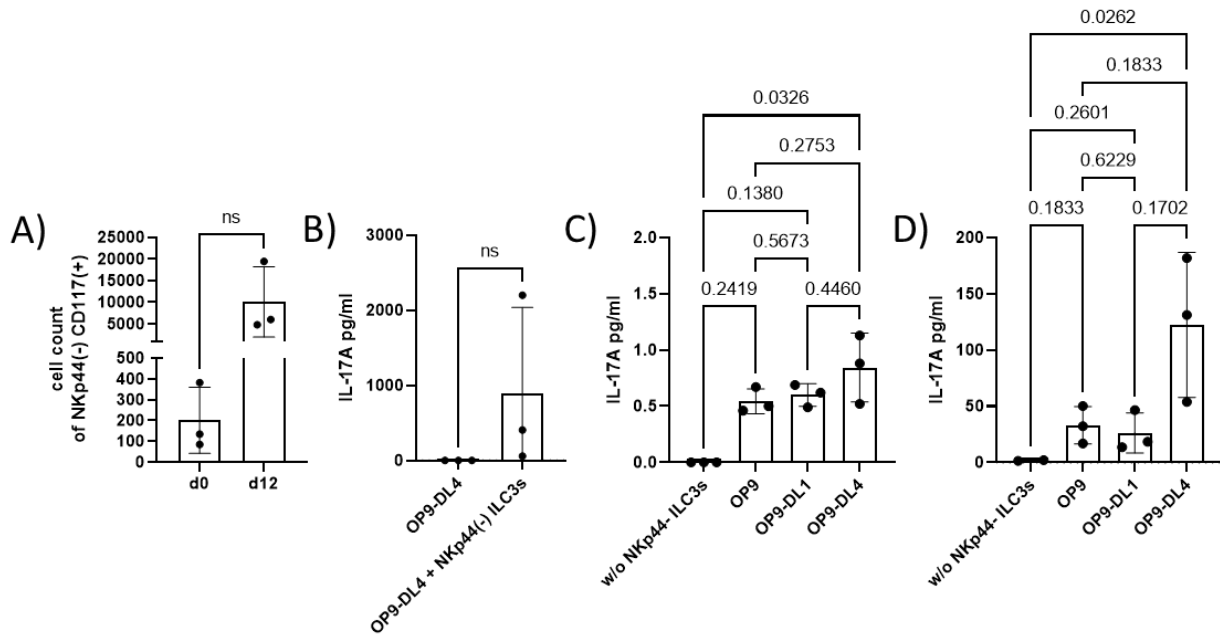
Fig. 25: Increased IL-17A production of NKp44⁻ ILC3s on OP9-DL4 stromal cells. (A) Cell count of sorted NKp44⁻ CD117⁺ ILC3s on day 0 and co-cultured with OP9-DL4 after stimulation with IL-1β, IL-23, IL-7 and IL-2 on day 12 (B) IL-17A production in the supernatant of sorted duodenal NKp44⁻ ILC3s co-cultured with OP9-DL4 after stimulation with IL-1β, IL-23, IL-7 and IL-2 on day 0 and 12. (C-D) IL-17A production in the supernatant of OP9-DL4 expanded duodenal (C) and of tonsil (D) NKp44⁻ ILC3s growing on OP9, OP9-DL1 and OP9-DL4 feeder cells after 3 days of stimulation with IL-1β, IL-23, IL-7 and IL-2. Error bars showing SD. Statistical significance analyzed by Wilcoxon test and KW test, corrected for multiple comparisons by controlling the FDR (Benjamini, Krieger and Yekutieli).

All together, these data suggest increased levels of IL-1β and IL-23 together with increased DL4 expression to be involved in duodenal accumulation of IL-17A-producing ILC3s in FAP.

3.5.2 Differentially expressed genes in the duodenal adenoma

After demonstrating that IL-17A-producing NKp44⁻ ILC3s are increased in FAP duodenal tissue, we next assessed how these cells might contribute to the development of adenomas. As data on the molecular mechanisms involved in duodenal adenoma formation in FAP patients is limited, we first performed bulk-RNA-seq analysis to identify differentially expressed genes (DEGs) in the normal and adenomatous mucosa of FAP duodenal tissue compared to normal mucosa of non-FAP controls.

Principal component (PC) analysis revealed significant differences between FAP adenoma (n=4) tissue, normal mucosa of FAP (n=4) and non-FAP (n=4) patients (Fig. 26).
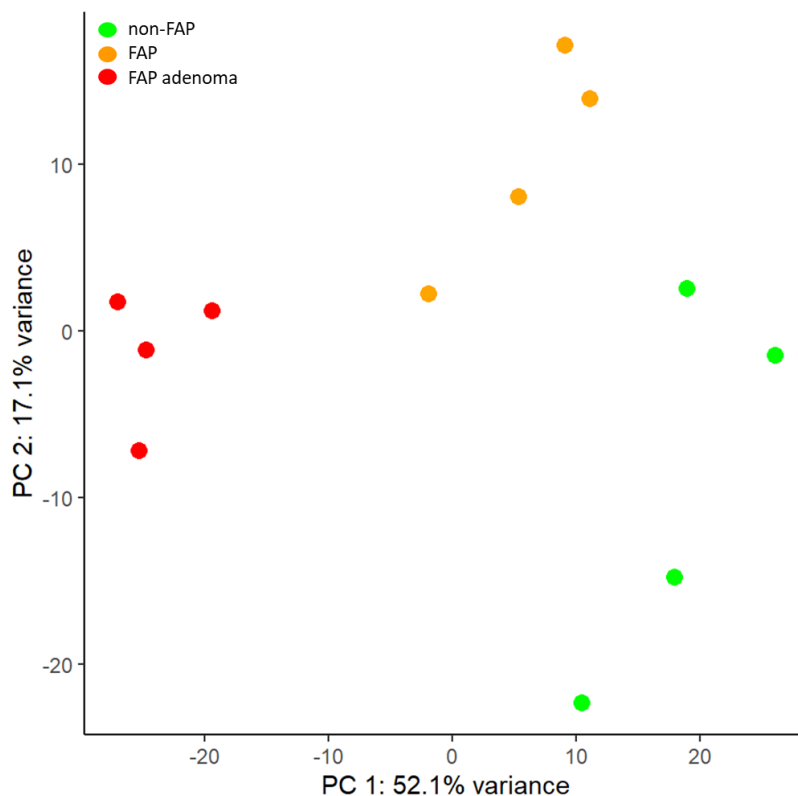
Fig. 26: FAP adenoma differs from normal mucosa of FAP and non-FAP patients. Principal component (PC) analysis was performed of bulk-RNA-seq data of duodenal normal (yellow) and adenomatous (red) mucosa of FAP and normal (green) mucosa of non-FAP patients. Data were normalized and batch-corrected prior to analysis. Each point represents the gene expression profile of a single sample. Figures created in cooperation with Emilia De Caro (DZNE, Bonn).

We identified a total of 183 genes that were significantly upregulated and 81 which were downregulated in FAP adenoma compared to control tissue. Comparing the FAP adenoma with the normal FAP mucosa, 117 genes were found to be upregulated and 34 genes displayed decreased expression.

A more detailed analysis revealed significant upregulation of several genes known to be involved in tumor formation and progression, including *CAPN8*, encoding for Calpain 8, a member of a family of intracellular calcium-activated neutral cysteine proteinases and involved in cancer initiation, progression, and metastasis (Zhong *et al.*, 2022; Storr *et al.*, 2011; Lan *et al.*, 2021); *TSPAN1*, encoding for Tetraspanin 1, which has been shown to promote growth of breast cancer cells via mediating PI3K/Akt pathway, to be involved in head and neck SCC and increased in CRC (Garcia-Mayea *et al.*, 2020; Wu *et al.*, 2021; Chen *et al.*, 2009); *CEMIP*, (Cell migration-inducing and hyaluronan-binding protein) which affects the Wnt and EGFR signaling pathways and is involved in the progression of various tumors including CRC and gastric cancer (Chen *et al.*, 2022; Fink *et al.*, 2015; Matsuzaki *et al.*, 2009); *KRT7* (Keratin 7) which has been shown to promote epithelial-mesenchymal transition in ovarian cancer (An *et al.*, 2021); *CDH3* (P-Caherin), *S100P* (calcium-binding protein) and *DUOX2* (dual oxidase 2) which have been found to be overexpressed in CRC (Kumara *et al.*, 2017; Wang *et al.*, 2012; Zhang *et al.*, 2021) (Fig. 27).
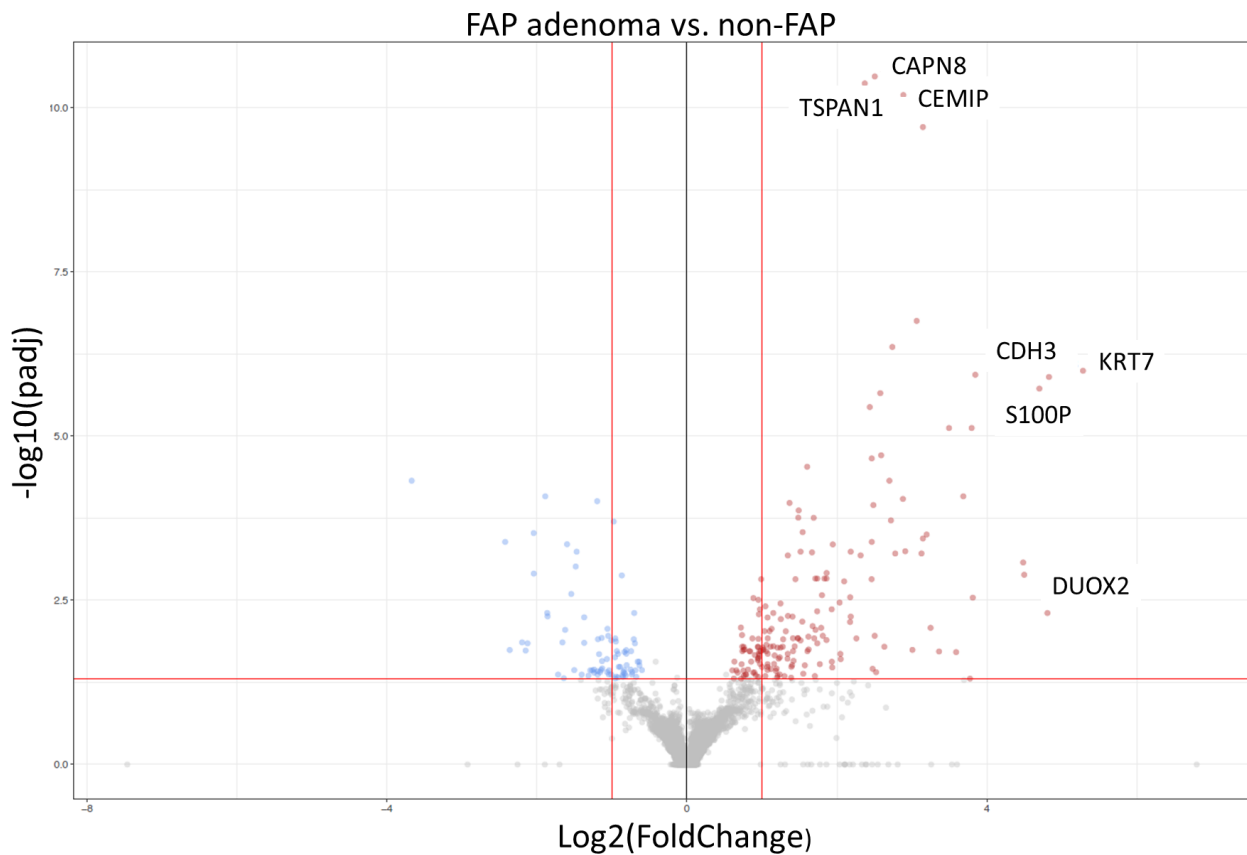
Fig. 27: Identification of differentially expressed genes in FAP adenoma compared to non-FAP controls. Dot plot of DEGs in FAP adenoma and non-FAP control tissue, based on bulk-RNA-seq data. Genes with a log2 fold change >1.5 or <-1.5 and an adjusted p-value <0.05 are considered differentially expressed and are highlighted in red or blue, depending on whether they are up- or downregulated. Figures created in cooperation with Emilia De Caro (DZNE, Bonn).

To confirm these results, mRNA expression of these genes was measured by qRT-PCR in duodenal adenoma (n=9), normal (n=17) tissue from FAP patients, and the normal mucosa of non-FAP controls (n=13). The DEGs (*CAPN8*, *TSPAN1*, *CEMIP*, *KRT7*, *CDH3*, *S100P* and *DUOX2*) were significantly increased in FAP adenoma tissue compared to normal mucosa of FAP and non-FAP control tissues, supporting the bulk-RNA-seq findings (Fig. 28). Additionally, *DUOXA2*, the maturation partner of *DUOX2*, also showed increased mRNA expression in FAP adenoma tissue (Fig. 28). *CDH3*, *DUOX2* and *DUOXA2* were significantly increased in normal FAP compared to normal non-FAP tissue (Fig. 28). Altogether, these findings suggested a role for *CAPN8*, *TSPAN1*, *CEMIP*, *KRT7*, *CDH3*, *S100P* and *DUOX2* in the duodenal adenoma formation in FAP patients.
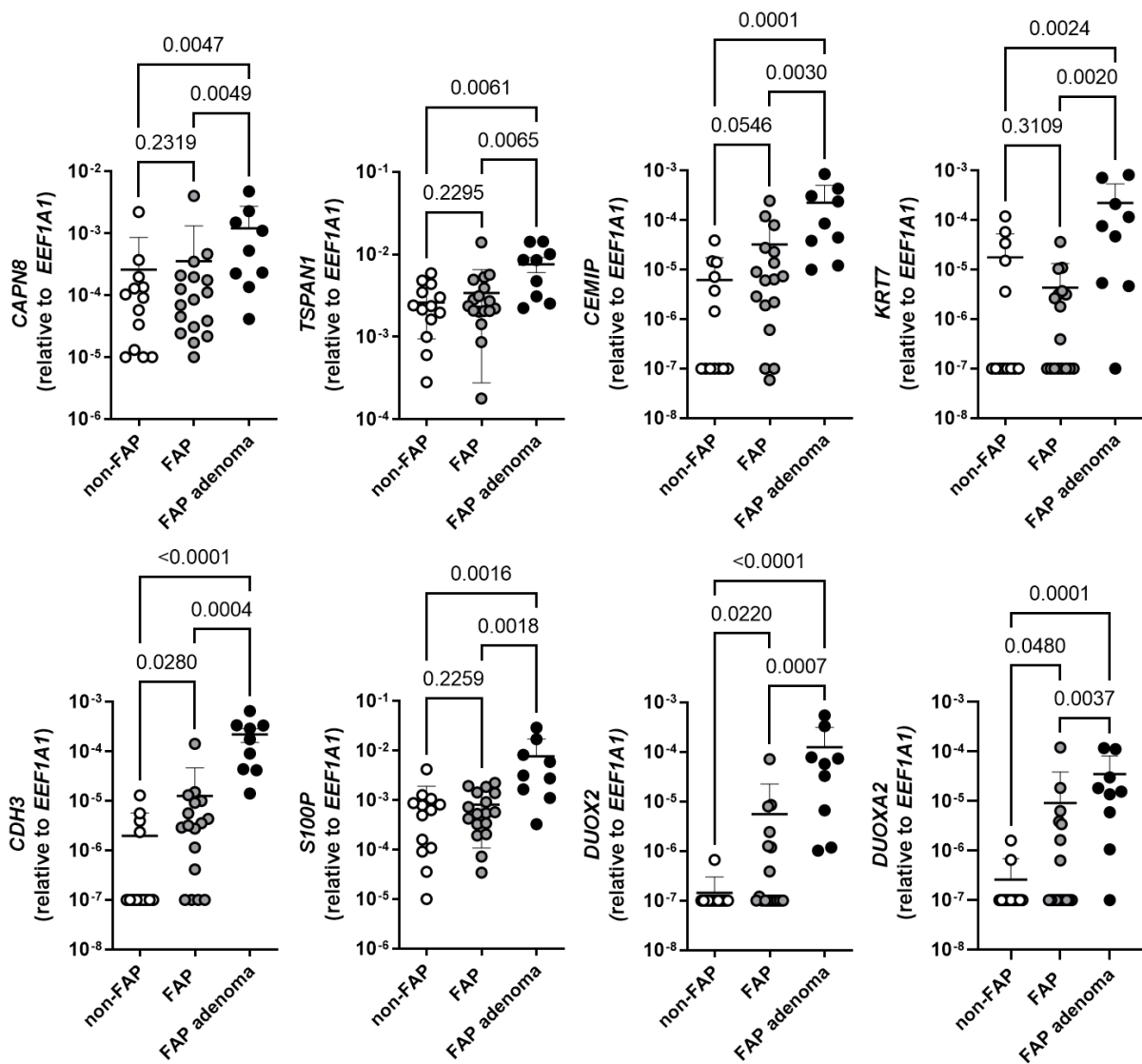
Fig. 28: Higher mRNA expression of specific bulk-RNA-seq DEGs in duodenal FAP adenomas. mRNA expression of *CAPN8*, *TSPAN1*, *CEMIP*, *KRT7*, *CDH3*, *S100P*, *DUOX2* and *DUOXA2* in duodenal adenomatous (n=9) and normal mucosa (n=17) of FAP and non-FAP patients (n=13). Error bars showing SD. Statistical significance analyzed by KW test, corrected for multiple comparisons by controlling the FDR (Benjamini, Krieger and Yekutieli).

3.5.3 IL-17A induces *DUOX2* and *DUOXA2* expression in duodenal organoids

Finally, we analyzed whether IL-17A-producing NKp44⁻ ILC3s might be involved in the transcriptional program of the DEGs and thus in promoting adenoma formation in FAP.

To this end, we generated duodenal organoids from normal duodenal mucosa (n=3) of FAP patients and non-FAP (n=3) controls (Fig. 29A).

The presence of duodenal cells, such as goblet and paneth cells, was confirmed through a combination of immunofluorescence analysis and qRT-PCR using Muc-2 (mucin-2) and LYZ (lysozyme) as markers, respectively (Fig. 29B/C). Additionally, qRT-PCRs were used to analyze the expression of *KI67* (marker for proliferating cells) and *LGR5* (marker for stem cells) in duodenal organoids (Fig. 29C). These analyses did not reveal any significant differences in the expression of these molecules between duodenal organoids of FAP patients and non-FAP controls (Fig. 29C).
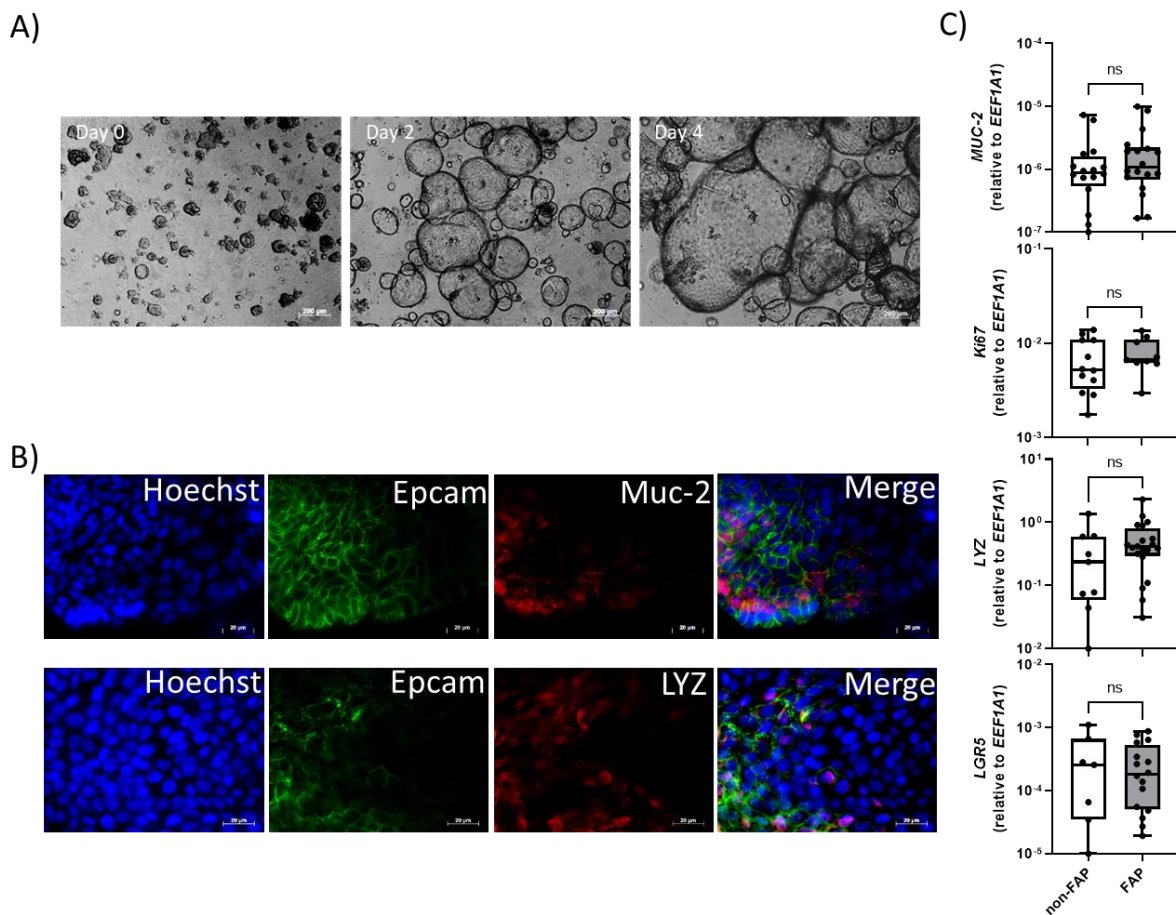
Fig. 29: Characterization of duodenal organoids. (A) Representative images of duodenal organoids on day 0, 2 and four (scale bar 200 μm). (B) Representative immunofluorescence images of duodenal organoids stained with Hoechst (nuclei), Epcam, Mucin-2 (Muc-2 for goblet cells) and lysozyme (LYZ for paneth cells) (scale bar 20 μm). (C) mRNA expression of *MUC-2*, *KI67*, *LYZ* and *LGR5* (for stem cells) in duodenal organoids of FAP (n=3) and non-FAP (n=3) controls. Error bars showing SD. Statistical significance analyzed by Mann-Whitney test.

Immune and stromal cells as potential cytokine-producers are absent in organoid cultures. Therefore, we could evaluate potential effects of recombinant IL-17A and IL-17A-producing NKp44⁻ ILC3s, respectively on duodenal epithelial cells using the established duodenal organoid model (Chen *et al.*, 2019a).

Thus, duodenal organoids were stimulated with 10 ng/ml recombinant human IL-17A after 5 days of culture. Following additional 20 h, organoids were then analyzed with respect to expression of genes differentially expressed in the bulk-RNA-seq analysis, namely *CAPN8, TSPAN1, CEMIP, KRT7, CDH3, S100P, DUOX2,* and *DUOXA2*. Interestingly, only *DUOX2* and its maturation factor *DUOXA2* were significantly elevated after IL-17A stimulation, whereas no such alterations were found for the other analyzed genes (Fig. 30).
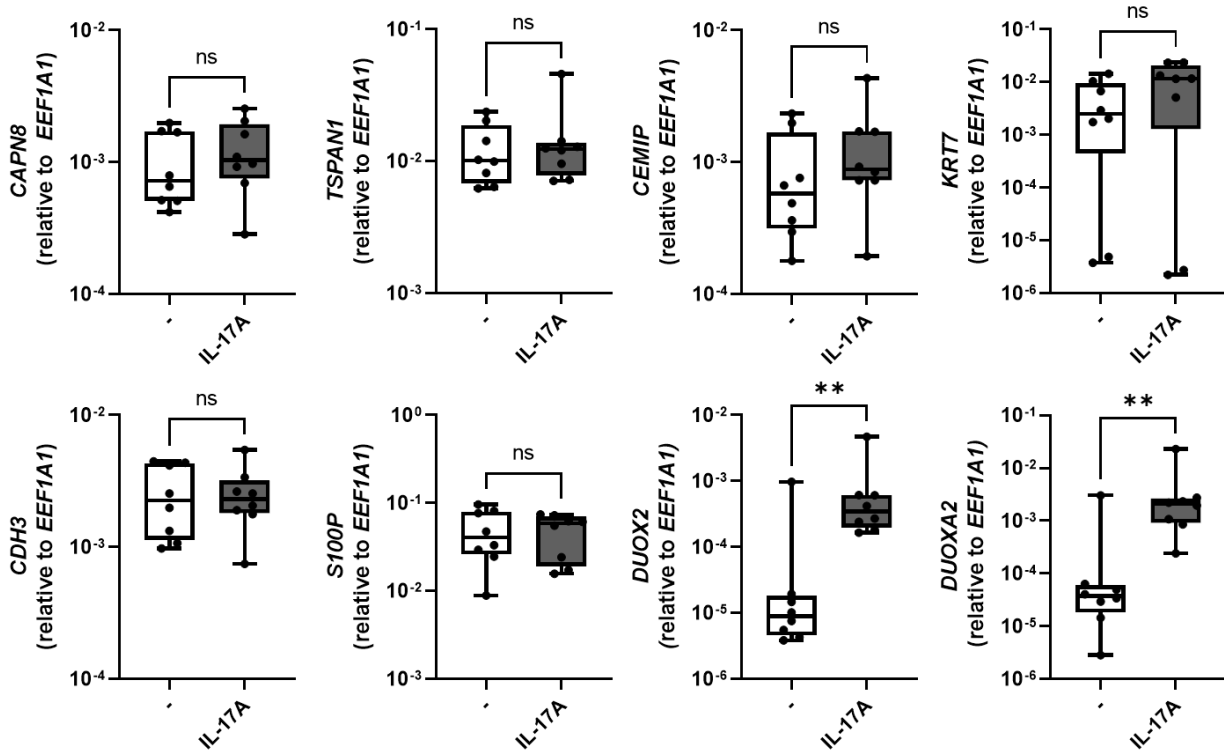
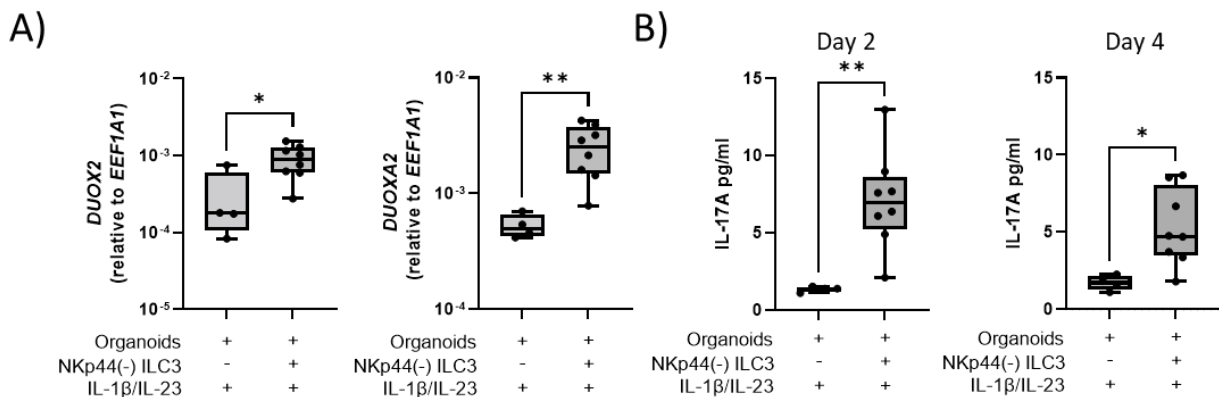

Fig. 30: IL-17A induces *DUOX2* and *DUOXA2* in duodenal organoids. mRNA expression of *CAPN8, TSPAN1, CEMIP, KRT7, CDH3, S100P, DUOX2* and *DUOXA2* in duodenal organoids (n=6) unstimulated and stimulated with IL-17A for 20 h. Error bars showing SD. Statistical significance analyzed by Wilcoxon test.

To further assess the impact of IL-17A-producing NKp44⁻ ILC3s on duodenal organoids, OP9-DL4 feeder cells were used to expand Lin⁻ CD127⁺ CD117⁺ NKp44⁻ ILC3s from normal duodenal mucosa of FAP patients.

After 12 days, the expanded ILC3s were sorted again, NKp44⁻ ILC3s co-cultured with duodenal organoids (n=4) in matrigel and stimulated every two days with recombinant IL-1β and IL-23 to activate ILC3s. After 4 days, the organoids were lysed and mRNA expression of *DUOX2* and *DUOXA2* was analyzed.

In line with our findings after stimulation with recombinant IL-17A, we observed *DUOX2* and *DUOXA2* levels to be significantly higher in organoids co-cultured with NKp44⁻ ILC3s than in organoid controls (Fig. 31A). Additionally, on day two and four, we found increased levels of IL-17A in supernatants from organoid co-cultured with NKp44⁻ ILC3s (Fig. 31B).

Fig. 31: Stimulated duodenal NKp44⁻ ILC3s induce *DUOX2* and *DUOXA2* mRNA expression in duodenal organoids. (A) mRNA expression of *DUOX2* and *DUOXA2* of duodenal organoids (n=4) co-cultured with or without NKp44⁻ ILC3s in presence of IL-1β and IL-23. (B) IL-17A in the supernatant of duodenal organoids co-cultured with or without NKp44⁻ ILC3s in presence of IL-1β and IL-23 after 2 and four days. Error bars showing SD. Statistical significance analyzed by Mann-Whitney test.

To confirm IL-17A being involved in NKp44⁻ ILC3s induced upregulation of *DUOX2* and *DUOXA2* mRNA expression and to verify specificity of our findings, we next cultured organoids (n=6) in supernatants of NKp44⁻ ILC3s expanded on OP9-DL4 in the presence or absence of an IL-17A blocking antibody. As is shown in Fig. 32, we found blocking of IL-17A with a specific antibody to prevent NKp44⁻ ILC3s induced increase in *DUOX2* and

DUOXA2 mRNA expression, whereas no such effects were observed for the isotype control, indicating an IL-17A mediated effect of NKp44⁻ ILC3s (Fig. 32).
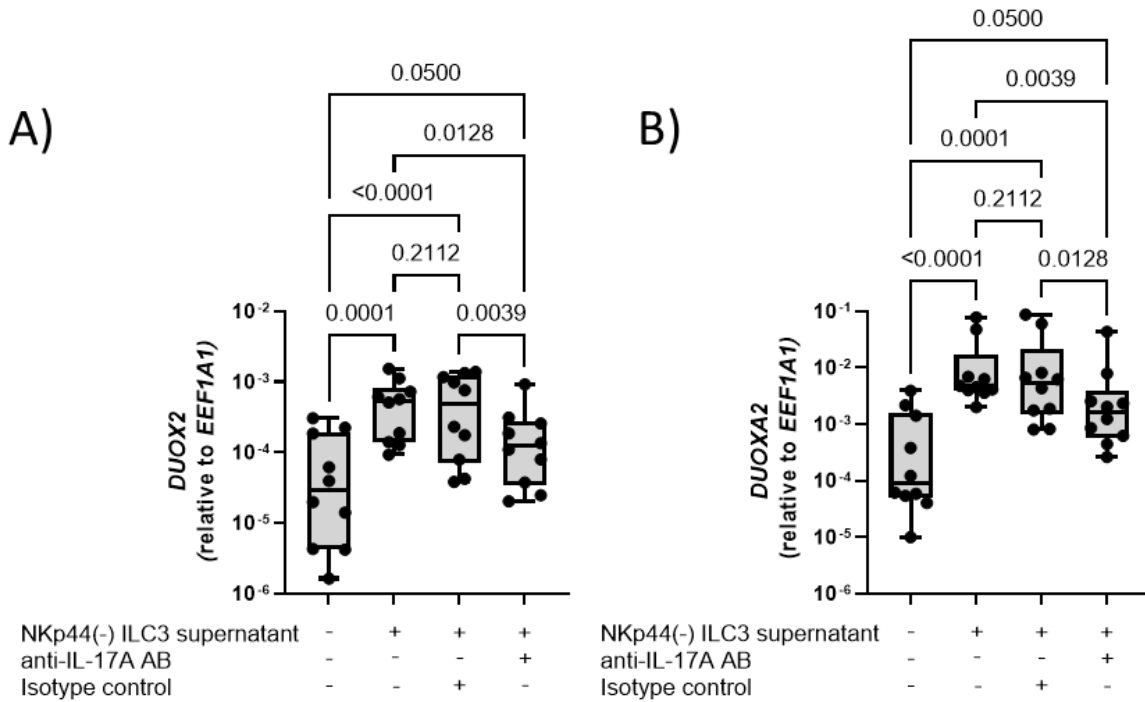
Fig. 32: Increased *DUOX2* and *DUOXA2* expression of duodenal organoids incubated with IL-17A supernatant of NKp44⁻ ILC3s. *DUOX2* (A) and *DUOXA2* (B) mRNA expression of duodenal organoids (n=6) that were incubated with IL-17A supernatant with or without IL-17A blocking antibody or isotype control. Error bars showing SD. Statistical significance analyzed by Friedman test, corrected for multiple comparisons by controlling the FDR (Benjamini, Krieger and Yekutieli).

Accordingly, after stimulating duodenal organoids (n≥3) with other ILC3-specific cytokines such as IL-22, TNF-α, IL-2, and IL-8 for 20 hours, an enhanced expression of *DUOX2* and *DUOXA2* was not observed (Fig. 33).
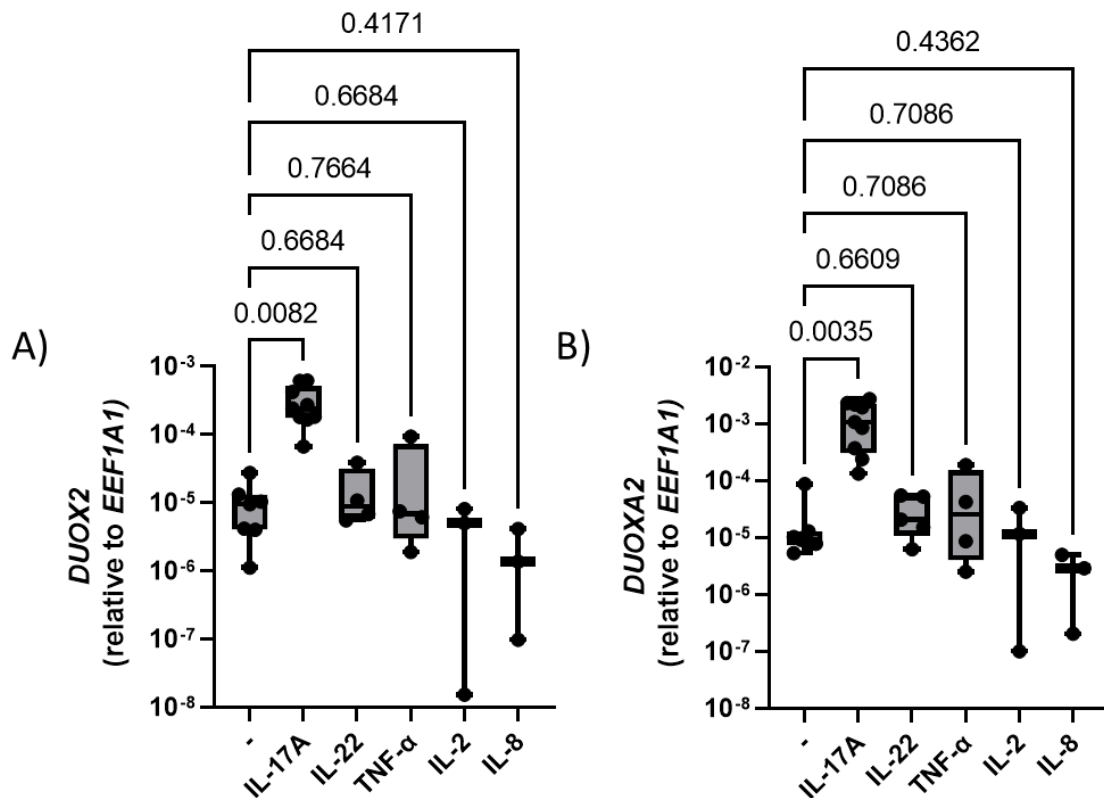
Fig. 33: Increased *DUOX2/A2* expression is IL-17A specific. *DUOX2* (A) and *DUOXA2* (B) mRNA expression of unstimulated and stimulated (IL-22, IL-17A, TNF-α, IL-2 and IL-8 (10 ng/ml)) duodenal organoids (n≥3) for 20 h. Error bars showing SD. Statistical significance analyzed by KW test, corrected for multiple comparisons by controlling the FDR (Benjamini, Krieger and Yekutieli).

Finally, we evaluated Duox2 expression on the protein level using immunofluorescence staining of duodenal adenomatous (n=3) and normal mucosa (n=3) tissue of FAP and non-FAP (n=3) patients, which confirmed upregulation of Duox2 in FAP adenomas, further supporting a role of Duox2 in the adenoma formation of FAP patients (Fig. 34).
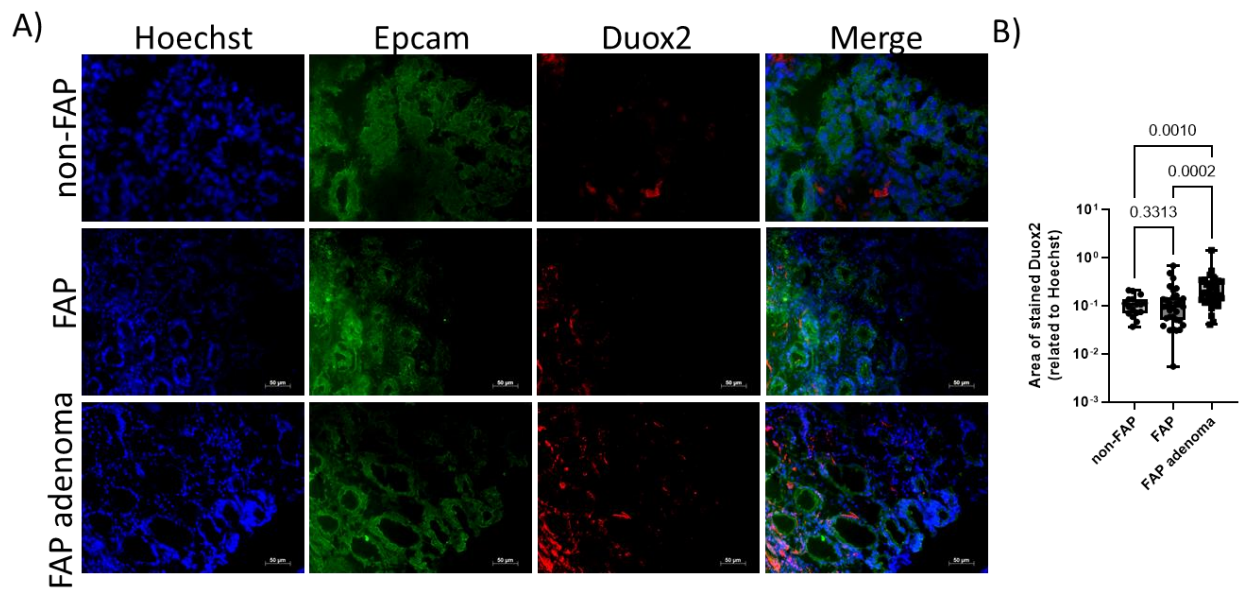
Fig. 34: Increased Duox2 in adenomatous tissue of FAP patients. (A) Representative immunofluorescence staining of adenomatous (n=3) and normal mucosa (n=3) of FAP and normal mucosa of non-FAP (n=3) patients with Hoechst (blue), Epcam (green) and Duox2 (red). (B) Percentages of stained Duox2 in three different patients per cohort. Various images (n≥17) were measured with the threshold of ImageJ and related to the measured threshold of Hoechst. Error bars showing SD. Statistical significance analyzed KW test, corrected for multiple comparisons by controlling the FDR (Benjamini, Krieger and Yekutieli).

4. Discussion

Familial adenomatous polyposis is an autosomal dominant inherited GI tumor syndrome caused by an *APC* mutation resulting in the development of numerous colonic adenomas which almost inevitably become CRC if prophylactic colectomy is not performed. After prophylactic colectomy, the duodenum is of particular clinical relevance, as the majority of FAP patients also develop duodenal adenomas resulting in a significantly increased risk of duodenal carcinoma.

Unfortunately, the management of duodenal polyposis remains challenging due to the high recurrence rate of duodenal adenomas (Richard *et al.*, 1997). Therefore, patients have to undergo regular endoscopic surveillance and close monitoring is mandatory and in the case of advanced Spigelman stages (SS IV), pancreatico-duodenectomy (Whipple's surgery) or pylorus-preserving duodenectomy may be the only options. However, both endoscopic treatment and duodenectomy are associated with procedure-associated risks and can be burdensome for patients. Therefore, there is a strong clinical need for the development of novel immune-modulating and/or pharmacological therapeutic approaches to reduce adenoma formation.

Thus, a more comprehensive understanding of the mechanisms contributing to adenoma formation is crucial.

The rather weak genotype-phenotype correlation in duodenal polyposis and the observation that patients with the same pathogenic *APC* variant may represent with different clinical courses underlines the importance of other - non-genetic - factors (Brosens *et al.*, 2005; Groves *et al.*, 2002b).

The local immune response may represent such an additional modulating factor as both innate and adaptive immune cells have been shown to regulate tumor formation (Gonzalez *et al.*, 2018).

Therefore, the aim of this project was to perform a detailed analysis of the duodenal immune infiltrate in normal and adenomatous mucosa of FAP patients in comparison to non-FAP controls.

The study identified elevated levels of total ILCs, especially ILC3s in duodenal adenomas and normal mucosa of FAP in comparison to normal mucosa of non-FAP patients. ILC3s in adenomas and normal mucosa of FAP patients produced more IL-17A compared to non-FAP controls. This increase was specific for NKp44^{-} ILC3s. Furthermore, we observed FAP to be associated with increased mRNA expression of genes that are optimal for ILC3 activation, growth and differentiation, namely *IL1B*, *IL23A*, *DL1* and *DL4* (Heinrich *et al.*, 2022; Müller and Romagnani, 2022). To further explore the potential role of IL-17A-producing ILC3s in human duodenal adenoma formation we next performed bulk-RNA-seq analysis which indicated a role for *DUOX2* and *DUOXA2,* which have been shown to promote cancer progression (Zhang *et al.*, 2021; Wang *et al.*, 2015). IL-17A and stimulated, IL-17A-producing NKp44^{-} ILC3s were found to induce the expression of *DUOX2* and *DUOXA2*.

The data suggest that IL-17A-producing NKp44^{-} ILC3s might play a role in the duodenal adenoma formation of FAP patients and may have a pro-tumorigenic effect. In the next chapters, these findings are discussed.

4.1   Identification of increased frequencies of NKp44^{-} ILC3s in normal and adenomatous mucosa of FAP patients

The immune cell microenvironment in duodenal normal and adenomatous mucosa in FAP patients has not been characterized in detail before. This study demonstrates that duodenal frequencies of CD4^{+} Th cells, CD8^{+} cytotoxic T cells, CD4^{+}CD8^{+} T cells, B cells, NKT and NK cells did not vary significantly between normal or adenomatous duodenal tissue from FAP patients and normal non-FAP tissue. Similar results have also been observed in a FAP mouse model (APC[Min/+]) initially described by Su *et al.* (Su *et al.*, 1992). These mice carry a heterozygous germ-line mutation at codon 850 of the *APC* gene (Su *et al.*, 1992), resulting in adenomas in the small intestine and colon. Subsequent studies observed frequency of CD4^{+}, CD8^{+} T and NK cells in the small intestinal LP and adenomas of APC[Min/+] to be similar to that observed in WT mice, whereas B cells were found to be decreased in SI adenomas of APC[Min/+] mice (Wang *et al.*, 2020b; Akeus *et al.*, 2014; Chae *et al.*, 2010; Tanner *et al.*, 2016). However, an altered tumor immunosurveillance

response of APC[Min/+] T cells and increased Tregs have been found indicating an involvement of CD4^{+} and CD8^{+} T cell subsets in the progression of FAP (Akeus *et al.,* 2014; Chae and Bothwell, 2015; Tanner *et al.,* 2016; Yang *et al.,* 2021a). Therefore, further investigations are necessary to determine whether human duodenal T cell subsets and NK cells play a role in the early development of duodenal adenomas. While the frequencies of the main lymphocyte populations were examined in the present study, it is possible that subsequent studies may reveal differences in numbers of specific subpopulations, functional capacity, or spatial distribution in the tissue, which all could affect adenoma development.

However, the present study observed frequencies of total ILCs, the innate counterpart of CD4^{+} Th cells to be significantly elevated in duodenal normal and adenomatous mucosa of FAP patients in comparison to normal non-FAP mucosa. Although the overall frequencies of ILC1s and ILC3s were significantly increased in the FAP mucosa, the increase in ILC3s was more substantial. As a result, the total duodenal ILC population in FAP patients, especially within adenomas was primarily composed of ILC3s, which were mainly NKp44^{-} ILC3s. ILC2s were rarely found in the duodenal mucosa of both FAP and non-FAP patients, which is in line with previous research conducted by our group (Krämer *et al.,* 2017). Upon activation by IL-1β, IL-23 or Ahr ligands ILC3s secrete cytokines including IL-22, IL-2, IL-8, TNF-α and IL-17A (Artis and Spits, 2015; Spits *et al.,* 2013; Vivier et al., 2018). The specific cytokine profile depends on the subtype of ILC3s, with NKp44^{-} ILC3s preferentially producing IL-17A, whereas NKp44^{+} ILC3s secrete IL-22 (Cella *et al.,* 2009; Hoorweg *et al.,* 2012). In the present study, we found duodenal NKp44^{-} ILC3s in the normal mucosa of FAP patients, and to an even greater extent in FAP adenoma tissue, to produce significantly more IL-17A compared to non-FAP controls after P/I stimulation whereas no such differences were observed for other ILC3-related cytokines such as IL-8, IL-2, IL-22 and TNF-α.

Regarding the mechanism(s) potentially involved in FAP-associated duodenal accumulation of IL-17A-producing NKp44^{-} ILC3s several scenarios can be envisioned.

First, increased duodenal migration of IL-17A-producing NKp44^{-} ILC3s to or impaired egress from the duodenum could explain our findings. However, ILCs are considered to be primarily tissue-resident cells (Gasteiger *et al.,* 2015) that circulate only to a limited

extent and, in particular, cytokine-producing mature ILC3s are almost absent in peripheral blood (Lim *et al.*, 2017). Moreover, the frequency and composition of the circulating ILC pool were not different between FAP patients and controls, which also argues against such a mechanism.

Alternatively, the underlying genetic mutation in the *APC* gene may directly affect ILC functions. Indeed, APC has been shown to modulate lymphocyte function. As an example, Agüera-González *et al.* (2017) found APC to ensure T cell receptor-triggered activation through Nuclear Factor of Activated T cells and demonstrated APC deficiency in mice to impair Treg differentiation and the acquisition of a suppressive phenotype (Agüera-González *et al.*, 2017). More recently, Mastrogiovanni *et al.* demonstrated APC to regulate T lymphocyte migration (Mastrogiovanni *et al.*, 2022). While we cannot entirely rule out the involvement of such a process, our observations that IL-8, IL-2, IL-22, and TNF-α production remained unaffected, along with the increased IL-17A production being specifically observed in the duodenum and not in the colon might rather argue against such a mechanism.

However, APC mutation and potentially other involved genetic co-factors might indirectly affect the ILC3 compartment via alteration of the local microenvironment. In line with this, Li *et al.* (2020b) found differentially expressed genes in the colonic normal mucosa of FAP patients compared to normal mucosa of patients with sporadic CRC and increased lymphocyte subsets in high grade adenomas and carcinomas of FAP patients indicating alterations in the microenvironment due to germline APC mutation (Li *et al.*, 2020b). FAP normal mucosa already exhibited enhanced metabolic processes and proliferative activity, which may be due to the long-term effects of inherited mutations in the *APC* gene (Li *et al.*, 2020b). Moreover, a neoepitope derived from a novel human germline APC gene mutation in FAP has been demonstrated to show selective immunogenicity and to induce a cytotoxic T cell response in controls but not in FAP patients suggesting that CD8^{+} T cells from individuals carrying this germline APC mutation have been tolerized to the mutation (Majumder *et al.*, 2018). Additionally, it is particularly interesting to note that changes in the mucosal microenvironment are also found in other hereditary cancer syndromes such as Lynch syndrome (LS) compared to non-LS control specimens, which further differs between tumor-free LS carriers and LS tumor patients (Bohaumilitzky *et al.*, 2022). The

alterations in the local microenvironment might also be possible due to microbial dysbiosis. In line with this, unpublished data of our group and Liang *et al.* (2020) demonstrated that *APC* mutation was closely related to changes of gut microbiota (Liang *et al.*, 2020). A direct interplay between the microbiome and FAP-associated ILC3 accumulation could not be studied so far.

ILCs have been shown to proliferate, differentiate and become activated in response to the local microenvironment (Gao *et al.*, 2017; Bernink *et al.*, 2015; Bal *et al.*, 2016). Moreover, there is compelling data demonstrating ILC subsets can convert their phenotype and function depending on the local microenvironment (Bernink *et al.*, 2015; Cella *et al.*, 2019; Vonarbourg *et al.*, 2010). Accordingly, frequencies and composition of the ILC compartment can vary depending on the tissue or organ-specific milieu (Simoni *et al.*, 2017; Simoni and Newell, 2018; Krämer *et al.*, 2017). Furthermore, disease-associated alterations of the microenvironment, as can be found in chronic inflammation, significantly affect ILC composition (Saez *et al.*, 2021; Bernink *et al.*, 2019; Bernink *et al.*, 2013). In line with this, IL-17A-producing NKp44$^-$ ILC3s have been found in various pathologies including cystic fibrosis (Golebski *et al.* 2019), psoriasis (Bernink et al., 2019) and inflammatory bowel diseases (Creyns *et al.*, 2020).

The local cytokine microenvironment plays a crucial role in this context. In particular, cytokines belonging to the common γ-chain family (IL-7, IL-15) are essential for the development of all known ILC subsets (Nagasawa *et al.*, 2018). IL-7 is critical for the development of ILC2 and ILC3 subsets, while IL-15 has an important role in developing ILC1s (Nagasawa *et al.*, 2018). In addition to IL-7 and IL-15, numerous cytokines have been found to regulate ILC differentiation and plasticity. For example, IL-12 and IL-18 have been demonstrated to drive plasticity from ILC2s and ILC3s in ILC1s (Bernink *et al.*, 2015; Cella *et al.*, 2019; Vonarbourg *et al.*, 2010), whereas IL-4 might promote the differentiation of human ILC3s and ILC1s in ILC2s (Bal *et al.*, 2016; Golebski *et al.*, 2019).

Interestingly, we observed duodenal mRNA levels of *IL23A* and *IL1B* to be increased in both normal and adenomatous biopsies of FAP patients. IL-23 and IL-1β are critical players in the immune system, particularly in the context of inflammatory responses and regulation of immune cells, shaping the immune response to various challenges, including infection and malignancy (Langowski *et al.*, 2006; Li *et al.*, 2012). IL-23 is a heterodimeric

cytokine composed of two subunits, p19 and p40, which belong to the IL-12 cytokine family (Oppmann *et al.*, 2000). It is mainly produced by antigen-presenting cells such as dendritic cells and macrophages (Oppmann *et al.*, 2000). IL-23 is known for its essential role in the maintenance and expansion of Th17 cells, a subset of Th cells involved in the pathogenesis of several autoimmune and inflammatory diseases (Cauli *et al.*, 2015). IL-1β, a member of the IL-1 family, is a potent pro-inflammatory cytokine synthesized predominantly as an inactive precursor by monocytes, macrophages and dendritic cells (Lopez-Castejon and Brough, 2011). Upon activation by inflammasomes, IL-1β is cleaved to its active form and secreted to orchestrate a variety of immune and inflammatory processes (Lopez-Castejon and Brough, 2011). Of note, both cytokines have been shown to contribute to the expansion and regulation of ILCs by driving the conversion of ILC1s and ILC2s to ILC3s and subsequently modulating mucosal immune responses (Bernink *et al.*, 2015; Cella *et al.*, 2009; Golebski *et al.*, 2019). In addition, IL-1β and IL-23 stimulate the production of IL-17A and IL-22 by ILC3s (Vivier *et al.*, 2018) suggesting a potential role for these cytokines in the development of FAP-associated adenomas.

In line with this, we observed a correlation between *IL23A* and *IL1B* mRNA levels and frequency of duodenal IL-17A-producing NKp44⁻ ILC3s. In addition, recombinant human IL-23 together with IL-1β induced expansion of duodenal NKp44⁻ ILC3s on OP9-DL4 feeder cells, confirming previous results of other groups (Lim *et al.*, 2017). Finally, we found stimulation of NKp44⁻ ILC3s cultured in the presence of OP9-DL4 feeder cells or duodenal organoids to induced IL-17A production. Altogether these findings suggested increased mucosal IL-23 and IL-1β, most likely together with additional alterations of the local microenvironment, to promote duodenal accumulation of IL-17A-producing NKp44⁻ ILC3s.

At present, the precise mechanisms underlying the elevated levels of IL-23 and IL-1β, as well as the specific cells implicated, remain unclear. As previously stated, IL-23 and IL-1β are generated by diverse immune cells, including dendritic cells and macrophages, in response to microbial pathogens and inflammatory cues (Geremia and Arancibia-Cárcamo, 2017). Consequently, it is plausible to hypothesize that FAP-associated modifications in the duodenal microenvironment, microbiome and/or disruptions in the

epithelial barrier may provoke corresponding inflammatory events. Nevertheless, further investigation is warranted in subsequent studies to confirm this hypothesis.

Alternatively, one could also consider the possibility that the increased mRNA levels of *IL23A* and *IL1B*, as well as accumulation of IL-17A-producing NKp44⁻ ILC3, are not causally implicated in FAP-associated adenoma development but rather represent a response to duodenal adenomatosis. However, elevated IL-17A-producing NKp44⁻ ILC3 frequencies were also observed in the duodenal mucosa of FAP patients without duodenal adenomas, which challenges such an explanation.

Interestingly, production of IL-17A in response to IL-1β/IL-23 stimulation was scarcely detectable in the absence of feeder cells or organoids, indicating additional factors to be important in this context. Upregulation of Notch ligands may represent such a factor. The Notch signaling pathway has been highly conserved throughout evolution and controls numerous developmental decisions, including cell fate, homeostasis, and survival, in various tissues during embryonic and adult life (Bray, 2016; Golub, 2021). It is a key pathway that enables communication between two neighboring cells to repeatedly regulate numerous developmental tasks, thereby participating in the development and differentiation of many immune cell populations and may also regulate their survival and functions (Golub, 2021; Yasutomo, 2017). Vertebrates have four distinct Notch receptors, designated Notch 1-4, that can interact with five known ligands, namely Delta-like (DL) 1, 3, 4, Jagged 1, and 2 (Golub, 2021).

Regarding ILCs, Notch signaling plays an essential role in maintaining intestinal homeostasis by regulating the differentiation and function of ILCs and is importantly involved in the activation, growth, and differentiation of ILC3s (Croft *et al.*, 2022; Golub, 2021).

Sustained activation of Notch signaling is required to maintain intestinal NKp44⁺ ILC3s identity (Chea *et al.*, 2016), whereas TGF-β signaling counteracts the effect of Notch signaling during NKp44⁻ ILC3 differentiation (Viant *et al.*, 2016). Regarding cytokine production of ILC3s, Notch signaling has been shown to indirectly affect IL-22 secretion in the intestinal mucosa, by regulating the maturation of ILC subsets in the tissue and balancing their abundance, rather than directly altering their secretion whereby Notch

signaling, together with Ahr, is required for the development of intestinal IL-22-producing NKp44^{+} ILC3s (Lee *et al.*, 2011; Qiu *et al.*, 2012; Viant *et al.*, 2016; Golub, 2021). However, the Notch signaling pathway may also affect IL-17A production. For example, it has been demonstrated that Notch signaling is responsible for ILC2s acquiring ILC3 features, as it can promote the expression of *RORC* leading to IL-17A secretion without impacting ILC2 functions (Golebski *et al.*, 2019; Zhang *et al.*, 2017).

We observed mRNA expression of the Notch ligands *DL4* and *DL1* to be increased in both normal mucosa and adenomas of FAP patients. More importantly, we found *DL4* and *DL1* mRNA levels to positively correlate with numbers of IL-17A-producing NKp44^{-} ILC3s, suggesting Notch signaling to be involved in FAP-associated increase of the ILC subset. Functional experiments indicated that specifically DL4 might be important in this context as only culturing of NKp44^{-} ILC3s in the presence of OP9-DL4 cells induced a significant upregulation of IL-17A, whereas no such effects were observed in the presence of OP9 or OP9-DL1 cells.

Altogether, these observations suggested Notch signaling together with IL-23 and IL-1β to be involved in duodenal expansion of IL-17A-producing NKp44^{-} ILC3s. This is in contrast to reports demonstrating these stimuli to promote expansion of IL-22-secreting NKp44^{+} ILC3s (Croft *et al.*, 2022; Rankin *et al.*, 2013). However, composition of ILC3 subsets *in vivo* depends on the balance between different signals in the local microenvironment. For instance, TGF-β has been shown to block Notch signaling, thereby increasing numbers of NKp44^{-} ILC3s (Viant *et al.*, 2016). Of note, we observed increased TGF-β mRNA expression in normal and adenomatous duodenal mucosa of FAP patients, which might suppress NKp44^{+} ILC3s expansion. In line with this, NKp44^{+} ILC3s of ILCs did not increase in normal and adenomatous mucosa of FAP patients which argues against a conversion of NKp44^{+} into NKp44^{-} ILC3s. As ILC1s of ILCs decrease, a conversion of ILC1s to NKp44^{-} ILC3s is much more likely. Accordingly, previous studies have reported a direct conversion from ILC1s to IL-17A-producing NKp44^{-}ILC3s with IL-23 (Liu *et al.*, 2019; Koh *et al.*, 2019), which could be relevant to the findings in this study. Further investigation using *in-vitro* culture of duodenal ILC subsets on OP9 feeder or normal or adenomatous FAP cells stimulated with IL-23 could provide additional insights into this conversion.

In conclusion, the normal and adenomatous microenvironment of the duodenum in FAP patients provides a perfect milieu for NKp44$^-$ ILC3 growth and activation. However, further research has to be performed to elucidate the mechanisms underlying the increased mRNA levels of *IL1B/IL23/DL4*. While IL-23 and IL-1β, along with Notch ligand DL4, are well-established factors involved in the expansion and activation of NKp44$^+$ ILC3s, the mechanisms behind the increased presence of NKp44$^-$ ILC3s in the normal and adenomatous mucosa of FAP patients and whether it involves a conversion from ILC1s to NKp44$^-$ ILC3s requires further investigation.

4.2 Duodenum-specific increase of IL-17A-producing NKp44$^-$ ILC3s

The composition and function of the ILC pool have been shown to be compartment-specific (Krämer *et al.*, 2017). A gradient exists within the GI tract, characterized by a decrease in the frequency of conventional NK cells from the proximal to distal GI tract, while the frequency of helper ILCs increases (Krämer *et al.*, 2017). Notably, the colon exhibits a particularly high prevalence of NKp44$^+$ ILC3s (Krämer *et al.*, 2017).

This compartmentalization is also observed in patients with FAP. In these individuals, over 90 % of colonic ILCs were identified as ILC3s, with the majority expressing NKp44. Interestingly, the differences between respective intestinal sections might extend to the processes involved in adenoma formation as we observed frequencies of total ILC3s and NKp44$^+$ ILC3s to be lower in FAP colonic adenomas compared normal mucosa of FAP and non-FAP patients, which was in sharp contrast to our findings in the FAP duodenum. These observations of reduced ILC3 numbers in colon adenomas parallel findings in sporadic CRC patients, where a decrease in ILC3 populations was identified within tumor tissue (Carrega *et al.*, 2020; Goc *et al.*, 2021; Qi *et al.*, 2021).

This data suggests that ILC3s might play a more protective role in the colon, potentially due to the distinct cytokine profile of colon ILC3s when compared to their duodenal counterparts. For instance, unlike in duodenal NKp44$^-$ ILC3s, increased IL-17A production was not observed in colonic NKp44$^-$ ILC3s.

The phenotypical and functional differences in ILCs between the duodenum and colon of FAP patients may be indicative of variations in the local microenvironment. Accordingly, increased mRNA levels of *IL23A, IL1B, DL1*, and *DL4* were detected in the duodenum of FAP patients, but not in colonic normal and adenomatous FAP mucosa. This finding implies that ILC3s may have a different role or be regulated differently in the colonic normal and adenomatous microenvironment of FAP patients. In line with the hypothesis, previous studies demonstrated that in sporadic CRC tissue, a conversion of ILC subsets may occur, resulting in an increase in ILC1s and a decrease in ILC3s, particularly NKp44^{+} ILC3s (Cella *et al.*, 2019; Goc *et al.*, 2021). In this context, ILC3 to ILC1 conversion has been proposed to be associated with a loss of the protective function of ILC3s in the CRC microenvironment, characterized by a lack of anti-T cell response due to a decreased interaction between ILC3s and T cells (Goc *et al.,* 2021).

Further investigation is required to better understand this context-dependent role of ILC3s in adenoma formation.

4.3   The role of IL-17A-producing NKp44^{-} ILC3s in duodenal adenoma formation

Our data suggest IL-17A-producing NKp44^{-} ILC3s to be involved in duodenal adenoma formation and thus, potentially in the establishment of duodenal cancer. Similar observation has been made in SCC (Koh *et al.*, 2019) and in a mouse model of HCC (Liu *et al.*, 2019). In line with our data, suggesting IL-23 induced increase in IL-17A-producing NKp44^{-} ILC3s being involved in duodenal adenoma formation, Chan *et al.* (2014) demonstrated that overexpression of IL-23 in wild-type (WT) mice induced duodenal tumorigenesis, while mice lacking the IL-23p19 chain displayed reduced intestinal tumor growth (Chan *et al.*, 2014; Grivennikov *et al.*, 2012). Of note, mice deficient in immune cells (RAG2^{-/-} x IL-2Rγc^{-/-}) were resistant to tumor formation triggered by IL-23, whereas tumor development did not differ between RAG1^{-/-} (deficient in mature T and B cells) and WT mice, suggesting a critical role for ILCs in the development of IL-23-induced duodenal tumors (Chan *et al.*, 2014). Moreover, the reduction in tumor numbers observed in both IL-17A^{-/-} mice following IL-23 overexpression and APC[Min/+] mice with an IL-17A^{-/-} genotype

further supports a pro-tumorigenic role for IL-17A-producing NKp44$^-$ ILC3s in FAP (Chae *et al.*, 2010). IL-17A is a pro-inflammatory cytokine that plays a role in host defense, promotes inflammation and recruits immune cells such as neutrophils to the site of infection (Jin and Dong, 2013). It has been implicated in various pathologies such as psoriasis (Bernink *et al.*, 2019) where dysregulation of IL-17A can contribute to chronic inflammation and tissue damage (Zhao *et al.*, 2020).

In cancer, IL-17A has been shown to contribute to tumor growth, angiogenesis, and metastasis (Yang *et al.*, 2014). The mechanisms by which IL-17A boosts tumors are thought to be diverse. In the HCC mouse model studied by Liu *et al.* (2019), IL-17A-producing NKp44$^-$ ILC3 inhibited CD8$^+$ T cell immunity by promoting lymphocyte apoptosis and limiting their proliferation, thereby boosting cancer development (Liu *et al.*, 2019). Alternatively, IL-17A may promote cancer growth by stimulating the production of pro-inflammatory cytokines and chemokines (Wang *et al.*, 2009), which can attract immune cells and promote tumor cell proliferation. In addition, IL-17A may also directly enhance tumor cell survival and invasion by activating signaling pathways that are involved in these processes (Liu *et al.*, 2022).

Our data suggest that induction of *DUOX2/DUOXA2* might represent an additional potential mechanism by which IL-17A produced by NKp44$^-$ ILC3s may promote duodenal adenoma formation. Duox2 is a member of the NOX family of NADPH oxidases and produces hydrogen peroxide (H_2O_2) when it forms a complex with its maturation factor, DuoxA2 (Bedard and Krause, 2007; Dang *et al.*, 2020). NADPH oxidases serve as the first barrier of the intestinal epithelium and take part in the innate immune response of the intestinal mucosa (Leto and Geiszt, 2006; Grasberger *et al.*, 2015; Dang *et al.*, 2020) but also play a role in the development of various carcinomas with elevated Duox2 expression being observed in liver cancer, pancreatic cancer, and prostate cancer (Lu *et al.*, 2011; Wang *et al.*, 2015; Wu *et al.*, 2013a; Lyu *et al.*, 2022; Cao *et al.*, 2021; Qi *et al.*, 2016; Zhang *et al.*, 2021). In addition, Duox2 was found to be highly expressed in CRC and to promote CRC cell invasion and metastasis by affecting the ubiquitination status of ribosomal protein uL3 (Zhang *et al.*, 2021). Moreover, Duox2 has also been shown to affect the response to GI cancer treatment (Kang *et al.*, 2018; Nguyen *et al.*, 2015).

We found recombinant human IL-17A as well as IL-17A-producing NKp44$^-$ ILC3s to specifically induce increased expression of *DUOX2/DUOXA2* in duodenal organoids, whereas no such effects were observed for IL-22, TNF-α, IL-2, and IL-8. Furthermore, we found blocking of IL-17A with a specific antibody to prevent upregulation of *DUOX2*, induced by supernatant of NKp44$^-$ ILC3s. *Ex vivo* analyses confirmed increase *DUOX2/DUOXA2* gene and protein expression in duodenal adenoma tissue in FAP, which is in line with earlier reports (Thiruvengadam *et al.*, 2019). Furthermore, a germline mutation in the *DUOX2* gene has been linked to adenomatous polyposis (Yang *et al.*, 2021b).

Altogether, these findings indicate IL-17A being involved in the regulation of Duox2 and suggest a role for Duox2/DuoxA2 in FAP-associated duodenal adenoma formation. Consistent with this hypothesis, previous research indicated IL-17A to trigger an upregulation of *DUOX2* expression in various cell lines (Wu *et al.*, 2019). Moreover, it has been demonstrated that IL-17A can induce *DUOX2* expression by interacting with its receptors, IL17RA and IL17RC (Wu *et al.*, 2013b). This interaction subsequently activates the nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways (Yan *et al.*, 2019). The p65 subunit of NF-κB is then able to bind to a canonical NF-κB binding site located on the human *DUOX2* promoter, which in turn induces *DUOX2* expression (Wu *et al.*, 2013b).

IL-17A induced increased *DUOX2* expression might lead to increased H_2O_2 production as has been observed in various cell lines (Dang *et al.*, 2020; Wu *et al.*, 2013b; Wu *et al.*, 2019; Wu *et al.*, 2022). H_2O_2 belongs to reactive oxygen species (ROS) and plays an important role in tissue homeostasis, cellular signaling, differentiation, and survival (Liu *et al.*, 2023). An increase in the cellular levels of H_2O_2 may play a key role in malignant transformation (López-Lázaro, 2007). H_2O_2 is known to be linked to enhanced apoptosis (Hirpara *et al.*, 2001), DNA damage, and mutations that cause oxidative stress and contribute to genomic instability, which in turn promotes the development of adenomas and the growth of tumors (Burgueño *et al.*, 2021; D'Errico *et al.*, 2008; Wu *et al.*, 2013b; Wu *et al.*, 2019; Lee *et al.*, 2002).

In conclusion, this study sheds light on the molecular mechanism of IL-23 induced IL-17A-producing NKp44$^-$ ILC3s in duodenal adenoma formation, and highlights the role of

IL-17A in the regulation of *DUOX2* expression. The findings provide new insights into the early stages of intestinal carcinogenesis and its potential therapeutic targets.

4.4   Outlook/Limitations

Several limitations of this study need to be addressed. First, the biopsy samples used were rather small and weight could not be determined. In addition, intestinal intraepithelial and LP cells were merged, making comparisons with other studies difficult. Flow cytometry analyses of ILCs and other lymphocyte subsets were performed based on CD45$^+$ cells rather than related to tissue weight or analyzed within tissue sections as done by Bohaumilitzky *et al*. (Bohaumilitzky *et al.*, 2022). In this study, quantitative analyses were performed using immunohistochemical staining to obtain accurate data on T cells subsets. However, this technique cannot be applied to ILCs due to the relative scarcity of these cells and because a variety of different surface markers are required to reliably identify ILCs. For future experiments, spatial transcriptomic and multiplexed imaging technologies such as Co-detection by indexing (CODEX) (Black *et al.*, 2021) should be applied to gain better insights into the spatial relationships between ILCs and other cells within the tissue. In the present study, we tested *in situ* characterization of ILCs using the Multi-Epitope-Ligand-Cartography (MELC) technology, staining 100 antigens on a single tissue section (Schubert *et al.*, 2006). However, ILCs remained hard to detect, as only a small portion of the tissue section could be analyzed.

To acquire more comprehensive information about smaller cell subsets, such as ILCs, Treg, and Th17 cells, and their contribution to adenoma formation, the duodenal immune infiltrate should be further investigated using advanced flow cytometry instruments that enable simultaneous analysis of a greater number of marker molecules, like the Sony ID7000^{TM} Spectral Analyzer.

We performed single cell RNAseq (scRNAseq) of sorted Lin$^-$ cells from duodenal normal, adenomatous of FAP, and normal mucosa of non-FAP patients to investigate the gene expression profile of ILCs and identify potential molecular mechanisms involved in the duodenal adenoma formation. However, the quality and quantity of the data was not

sufficient for a robust analysis, and contamination with T and B cells occurred. Therefore, scRNAseq should be performed using purified ILCs or even better purified ILC3s. Since ILCs are present in low numbers in small biopsies, pooling of ILCs from a large number of biopsy samples may be necessary to obtain valid measurements. Utilizing duodenum resectates could help increase the number of ILCs/ILC3s, although implementing this approach is challenging, given that only a small percentage of FAP patients with SS IV undergo duodenectomy or Whipple surgery. ScRNAseq of ILC3s in colon, colonic adenomas, or CRC samples (Qi *et al.*, 2021; Wang *et al.*, 2021) has already been conducted, making it worthwhile to compare these findings with scRNAseq data from duodenal and colonic adenoma and tumor samples in FAP patients.

At present, endoscopic surveillance and polypectomy, along with duodenectomy for patients with extensive duodenal polyposis, serve as the standard approach to prevent duodenal cancer development. Additionally, colectomy is performed in nearly all FAP patients to reduce the risk of colon cancer. Considering the risks associated with these procedures and the potential post-operative decline in quality of life, numerous clinical trials have been conducted to evaluate drug-induced reduction of polyp burden, with the primary focus of these trials being the treatment of colonic adenomas (Giardiello *et al.*, 1993; Giardiello *et al.*, 2002; Cruz-Correa *et al.*, 2002; Cruz-Correa *et al.*, 2006; Steinbach *et al.*, 2000). Even if some of these studies reduced the number of colonic adenomas, the effect was only partial and a prophylactic colectomy was still recommended in the study participants (Giardiello *et al.*, 1993; Samadder *et al.*, 2016; Giardiello *et al.*, 2002; Steinbach *et al.*, 2000; Cruz-Correa *et al.*, 2002; Ulusan *et al.*, 2021). Regarding duodenal polyposis, Sulindac, a non-steroidal anti-inflammatory drug, did not display any significant effect on duodenal adenoma formation although some authors discussed its potential use as an additional treatment following endoscopic polyp excision (Burke *et al.*, 2020; Burke *et al.*, 2016; Nugent *et al.*, 1993; Richard *et al.*, 1997). Other trials testing celecoxib (COX-2 inhibitor), erlotinib (EGFR inhibitor) or a combination of erlotinib and sulindac observed some reduction in duodenal polyp burden (Samadder *et al.*, 2016; Samadder *et al.*, 2022; Phillips *et al.*, 2002; Ulusan *et al.*, 2021). However, these effects have only been investigated over short periods, and it remains uncertain whether they genuinely prevent

cancer development in these individuals. Consequently, further research and insight into the tumor growth process in FAP are necessary.

The findings of the present study indicate IL-23-induced increase in IL-17A-producing NKp44$^-$ ILC3s to play a role in the duodenal adenoma formation, which implies ILC3s, IL-17A and/or IL-23 might represent putative targets for chemoprevention of duodenal adenoma and carcinoma formation. Accordingly, there are already several clinical trials targeting ILC-related factors in other diseases, such as IBD (Cobb and Verneris, 2021). Guselkumab, a human immunoglobulin monoclonal antibody against the p19 subunit of IL-23, is effectively used in psoriasis patients and has been tested in IBD patients with promising results (Sandborn *et al.*, 2022; Howell *et al.*, 2018). Based on these promising results and the established role of the IL-23/IL-17A/JAK/STAT3 pathway in GI cancers (Owen *et al.*, 2019; Huynh *et al.*, 2017; Iwakura and Ishigame, 2006; Gaffen *et al.*, 2014), a clinical trial using guselkumab has also recently been performed in FAP patients (NCT03649971, 2018-2022). Although the final results of this trial have yet to be reported, it is tempting to speculate that suppressing the IL-23 signaling pathway should at least in part prevent accumulation and activation of IL-17A-producing NKp44$^-$ ILC3, thereby positively affect duodenal adenoma formation. In line with this hypothesis, Creyns *et al*. (2020) observed reduced frequencies of NKp44$^-$ ILC3s but an increase of NKp44$^+$ ILC3s in colonic and ileal tissue of IBD patients following treatment with ustekinumab (IL-12/IL-23 inhibitor) (Creyns *et al.*, 2020). Nevertheless, it remains unclear whether this observed effect is solely attributed to IL-23 inhibition, or if IL-12 blockade may also play a role. Additionally, it would be worthwhile to investigate if similar alterations are detectable in the duodenal mucosa. Thus, future studies examining duodenal and rectal/pouch ILCs in FAP patients following guselkumab treatment, could provide valuable insights. However, when considering potential ILC3/IL-17A targeting studies, it is crucial to recognize the complex nature of IL-17A effects, as anti-IL-17A treatment could potentially result in negative consequences. Clinical trials using the anti-IL-17A monoclonal antibodies brodalumab and secukinumab in IBD patients revealed a worsening of the disease phenotype (Fauny *et al.*, 2020; Hueber *et al.*, 2012). Hueber *et al.* (2012) hypothesized that IL-17A blockade might disrupt its protective function within the intestine, suggesting that targeting IL-23 may be a more promising approach (Hueber *et al.*, 2012).

In summary, this study demonstrates that IL-23/IL-1β induced IL-17A production of NKp44⁻ ILC3s can induce *DUOX2* expression and might promote the duodenal adenoma formation in FAP patients. Blocking of ILC3-targeting factors might be a putative target for clinical trials in FAP patients.

5. Abstract

Familial adenomatous polyposis (FAP) is an inherited gastrointestinal tumor syndrome. Apart from colonic polyposis and colorectal cancer, the occurrence of duodenal adenomas is the most common intestinal manifestation of FAP, resulting in a significantly increased risk of developing duodenal cancer compared to the general population. Only a proportion of FAP patients develop duodenal adenomas and the extent of duodenal polyposis varies considerably. Even within carriers of the same genetic variant of the adenomatous polyposis coli (*APC*) gene, duodenal phenotype and clinical course vary, indicating that, in addition to the genotype, other factors such as the local immune system play a role.

Here, we analyzed the potential role of innate lymphoid cells (ILCs) in the duodenal adenoma formation in FAP. Intestinal lymphocytes were isolated from normal and adenomatous tissue samples of 90 FAP and 35 non-FAP patients obtained during routine endoscopy and analyzed regarding phenotype and function by flow cytometry. Mucosal mRNA levels were assessed by qRT-PCR and bulk-RNA-seq. Furthermore, duodenal organoids were generated to analyze ILC function during adenoma formation.

Frequency of total ILCs was significantly increased in normal duodenal mucosa in FAP patients compared to controls and was highest in FAP adenoma tissue. This was especially true for group 3 ILCs (ILC3s). More importantly, we found FAP to be associated with an increased IL-17A production of duodenal NKp44$^-$ ILC3s. No such findings were made regarding colon ILC3s in FAP, indicating a duodenum-specific effect. Cytokines such as IL-1β and IL-23A and the Notch ligand Delta-like 4 (DL4) have been shown to be important in the regulation of intestinal ILC3 differentiation, maturation and function. We found *IL1B*, *IL23A*, and *DL4* mRNA expression to be significantly elevated in FAP duodenal adenoma and normal tissue and to correlate positively with the frequency of IL-17A$^+$ NKp44$^-$ ILC3s. Accordingly, we observed culturing NKp44$^-$ ILC3s on DL4 expressing OP9 feeder cells (OP9-DL4) in the presence of IL-1β/IL-23 to significantly increase ILC3's IL-17A production. Furthermore, bulk-RNA-seq and qRT-PCR revealed that duodenal adenomas of FAP patients had higher levels of dual oxidase 2 (*DUOX2*) and its maturation factor *DUOXA2,* which can promote cancer due to increased H_2O_2

production. Functional studies demonstrated both recombinant IL-17A and activated NKp44⁻ ILC3s to induce expression of *DUOX2* and *DUOXA2* in duodenal organoids.

These results suggest IL-17A-producing NKp44⁻ ILC3 to be involved in duodenal adenoma formation in FAP patients.

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8. References

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