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The role of obesity in macrophage-mediated mechanisms promoting early-onset colon cancer.

Katharina Marietta Scheurlen
University of Louisville

THE ROLE OF OBESITY IN MACROPHAGE-MEDIATED MECHANISMS PROMOTING EARLY-ONSET COLON CANCER

By

Katharina Marietta Scheurlen
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Katharina Marietta Scheurlen
M.D. - University of Heidelberg, Germany 2015

A Dissertation Approved on

March 31st, 2022

By the following Dissertation Committee:

_____
Susan Galandiuk, M.D.

_____
Irving G. Joshua, Ph.D.

_____
Dale A. Schuschke, Ph.D.

_____
Jun Yan, MD, Ph.D.

_____
Aruni Bhatnagar, Ph.D., FAHA

_____
Claudio Maldonado, Ph.D.

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ABSTRACT

THE ROLE OF OBESITY IN MACROPHAGE-MEDIATED MECHANISMS PROMOTING EARLY-ONSET COLON CANCER

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Early-onset colon cancer (EOCC) is a leading cause of cancer death among people younger than 50 years of age in the United States and is associated with metabolic dysfunction and obesity. Anti-inflammatory tumor-associated macrophages (TAM) and low Peroxisome Proliferator Activated Receptor Gamma (PPARγ) gene expression in colon cancer (CC) tissue promote tumor progression and decreased patient survival. Obesity-related hormones, such as leptin and adiponectin, have the potential to affect gene expression in TAM to promote CC progression and thereby link obesity and EOCC. The aim of this project was to identify target genes in human CC and to investigate the effects of leptin, adiponectin and the inflammatory macrophage metabolite itaconate on age- and obesity-related gene expression.

These studies have provided the following results:

1. M2-like macrophages created using our 14-day cell line model represent a distinct anti-inflammatory macrophage phenotype. Marker gene expression, protein expression and cell surface marker analyses revealed an M2-like expression profile similar to gene expression patterns described in human primary monocyte-derived macrophages with tumor-associated marker expression. This model provides a basis for in vitro investigation of anti-inflammatory mechanisms in cancer development.
2. The macrophage-specific enzyme is responsible for production of carcinogenic itaconate is encoded by Immune-Responsive Gene 1 (IRG1), which expressed in CC and is associated with decreased patient survival. As a macrophage metabolite affecting inflammation, itaconate may have a particular immunotherapeutic role in patients with CC and obesity, specifically with EOCC.
3. In M2-like anti-inflammatory macrophages, Itaconate downregulates PPARγ as a tumor suppressing factor and upregulates anti-inflammatory cytokines. Itaconate provides a link between obesity and CC and may thereby be a key regulator in EOCC.
4. M0 macrophages show limited responses to leptin and itaconate compared to M2-like cells, suggesting that the investigated mechanisms play a role in tumor progression rather than cancer onset.

5. Leptin and adiponectin mediate tumor-promoting mechanisms through macrophages, altering gene expression of the NOTCH4-GATA4-IRG1 axis. Upregulation of the genes GATA-binding factor 4 (GATA4), Delta Like Canonical Notch Ligand 4 (DLL4), Hes Related Family BHLH Transcription Factor With YRPW (HEY1), Notch Receptor 4 (NOTCH4), Serpin Family E Member 1 (SERPINE1) and Vascular Endothelial Growth Factor A (VEGFA) in CC is age-dependent, increased in the young and associated with decreased patient survival.

6. Upregulation of ATP Binding Cassette Subfamily G Member 5 (ABCG5) and GATA5 was obesity-dependent in CC, increased in the obese and associated with decreased patient survival.

Our data provide evidence that the obesity-related hormones leptin and adiponectin as well as the macrophage-specific metabolite itaconate can exert tumor promoting effects through metabolic reprogramming of macrophages by altering gene expression of the NOTCH4-GATA4-IRG1 axis.

Future studies investigating the function of this axis in macrophages and CC cells in the surgical patient are required. The NOTCH4-GATA4-IRG1 axis may provide a specific treatment target for young patients with EOCC.

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CHAPTER I

INTRODUCTION

Colorectal cancer (CRC) is one of the most common causes of cancer-related death in the USA (1). The rising incidence of obesity and metabolic dysfunction is accompanied by an increasing number of patients that are diagnosed with early-onset CRC (EOCRC) (2-5). EOCRC is generally defined as CRC occurring in individuals <50 years of age. Incidence rates in the United States increased by 22% from 2000 to 2013 and mortality increased by 13% (6). Regular screening for CRC has been recommended by the U.S. Preventive Services Task Force (USPSTF) beginning at age 50, which changed in the year 2021 (7). Due to the large-scale screening programs in these patients >50 years of age with removal of precancerous polyps during colonoscopy and earlier detection of CRC, the incidence and mortality of CRC has declined (8, 9). The increasing number of colon cancer (CC) cases among younger adults has, however, led to the recommendation of an earlier start of regular screening by the American Cancer Society (ACS) in 2018 and the USPSTF in 2021, beginning at age 45 (10, 11). The parallel increase in the incidence of obesity in the young and EOCRC suggests that obesity-related inflammatory mechanisms may play a greater role in the development of CRC than assumed.

Most cases of CRC are either sporadic in etiology, based on a somatic mutation involving adenomatous polyposis coli gene (APC) function, or less often hereditary, as in hereditary nonpolyposis CRC (HNPCC) or familial adenomatous polyposis (FAP) (12, 13). HNPCC is the most common cause of hereditary CRC (14). While the proportion of other hereditary cancer syndromes in patients with EOCRC is widely unknown, HNPCC represents 4%-13.5% of cancers in patients with EOCRC (14). More than 80% of patients with EOCRC, however, are diagnosed with sporadic cancers (14).

Only a small percentage of CRC is actually associated with inflammatory bowel disease such as ulcerative colitis or Crohn's disease (15, 16). Compared to 0.4% of patients with a late onset of CRC, young patients with CRC showed an increased prevalence of inflammatory bowel disease in 3% of cases (17). This suggests that inflammatory processes may have a greater role in the onset and development of CRC than previously assumed. A systemic proinflammatory state and local immune mechanisms on a tissue level may link obesity to early cancer onset and tumor progression in EOCRC (18).

As essential components of the immune inflammatory response, macrophages are able to orchestrate inflammatory mechanisms and therefore tumorigenesis. The tumor microenvironment (TME) is a dynamic environment surrounding the tumor and a critical part of these regulatory processes. Tumor cells, immune cells and the blood and lymphatic vascular networks interact with stromal cells and their extracellular matrix, coordinating cancer establishment, tumor growth and metastasis (19). Current evidence shows that tumor-associated macrophages

(TAMs) play a central role in the dynamic processes within the TME, contributing to tumor promoting effects as well as contributing to tumor suppressing mechanisms in CRC (19). Various functions require dynamic switching between different TAM phenotypes. These phenotypes depend upon specific metabolic pathways within TAMs, which provide a source for functional metabolites and phenotype-specific cytokine expression, mediating inflammatory activities during cancer development (20, 21). The impact of TAM-mediated effects within the TME on cancer progression and a patient's outcome is poorly understood.

The purpose of this work is to develop a coculture model of TAM and CC cells and to investigate the effects of obesity-related inflammatory signaling pathways. A specific focus is the anti-inflammatory mediator itaconate, a macrophage-specific metabolite involved in cancer progression.

To give an overview of the various genes and macrophage markers discussed in this work, **Table 1** provides a list of abbreviations.

Table 1

List of abbreviations

ABCG	ATP Binding Cassette Subfamily G Member	**LPS**	Lipopolysaccharide
ACOD1	Cis-Aconitate Decarboxylase	**Ly6C**	Lymphocyte antigen 6 Complex
ACS	American Cancer Society	**MAPK**	Mitogen-Activated Protein Kinase
ADAMTS1	ADAM Metallopeptidase With Thrombospondin Type 1 Motif 1	**MGLL**	Monoacylglycerol Lipase
ADIPOR	Adiponectin Receptor	**MHC**	Major Histocompatibility Complex
AKT	AKT Serine/Threonine Kinase	**MMP**	Matrix Metalloproteinase
AMPK	AMP-activated Protein Kinase	**MMR**	DNA Mismatch Repair
AP-1	Activating Protein-1	**mTOR**	Mechanistic Target Of Rapamycin kinase
APC	Adenomatous Polyposis Coli	**NASH**	Non-alcoholic Steatohepatitis
ARG1	Arginase 1	**NFLD**	Non-alcoholic Fatty Liver Disease
ATP	Adenosine Thiotriphosphate	**NFκB**	Nuclear Factor Kappa B
BMI	Body Mass Index	**NICD**	Notch Intracellular Domain
B-Raf	B-Raf Proto-Oncogene	**NOTCH**	Notch receptor
CC	Colon Cancer	**NRF**	Nuclear factor erythroid 2-related Factor 2
CCL	CC chemokine Ligand	**NSAID**	Nonsteroidal Anti-inflammatory Drugs
CCR	CC chemokine Receptor	**OXPHOS**	Oxidative Phosphorylation
CD	Cluster of Differentiation	**P53**	Tumor protein p53
CMS	Consensus Molecular Subtype	**PBP**	(PPAR)-binding protein
COX	Cyclooxygenase	**PDGF**	Platelet-derived Growth Factor
CRC	Colorectal Cancer	**PFKFB3**	6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 3
CSF	Colony Stimulating Factor	**PI3K**	Phosphoinositide Kinase-3
CSL	CBF1, Suppressor of Hairless, Lag-1	**PMA**	Phorbol 12-Myristate-13-Acetate
CXCL	C-X-C motif Chemokine Ligand	**PPARγ**	Peroxisome Proliferator Activated Receptor Gamma

CXCR	C-X-C motif Chemokine Receptors	**RNA-seq**	RNA sequencing
DLL4	Delta Like Canonical Notch Ligand 4	**RNI**	reactive nitrogen intermediates
EMT	Epithelial-Mesenchymal Transition	**ROI**	Reactive Oxygen Intermediates
EOCC	Early-Onset Colon Cancer	**ROS**	Reactive Oxygen Species
EOCRC	Early-Onset Colorectal Cancer	**SERPINE1**	Serpin Family E Member 1
FAP	Familial Adenomatous Polyposis	**SIGN**	Specific Intercellular adhesion molecule-grabbing Nonintegrin
GATA	GATA-binding protein	**STAT3**	Signal Transducer and Activator of Transcription 3
GLP	Glucagon-Like Peptide	**TAM**	Tumor-associated macrophage
HES	Hairy and Enhancer of split	**TCA cycle**	Tricarboxylic Acid cycle
HEY	Hes Related Family BHLH Transcription Factor With YRPW	**TGF**	Transforming Growth Factor
HIF1α	Hypoxia Inducible Factor 1 Subunit Alpha	**TIMP**	Tissue Inhibitor of Metalloproteinase
HNPCC	Hereditary Non-polyposis Colorectal Cancer	**TLR**	Toll Like Receptor
IFN	Interferon	**TME**	Tumor Microenvironment
IL	Interleukin	**TNF**	Tumor-Necrosis Factor
IL-1Ra	Interleukin-1 Receptor Antagonist	**USPSTF**	U.S. Preventive Services Task Force
IRF	Interferon-Regulatory Factor	**VEGF**	vascular endothelial growth factor
IRG1	Immune-Responsive Gene 1	**WHO**	World Health Organization
JAK	Janus kinase	**WNT**	WNT family member
K-Ras	KRAS Proto-Oncogene		

CHAPTER II

SIGNIFICANCE OF OBESITY-RELATED INFLAMMATION IN EARLY-ONSET COLON CANCER

a. Background

Overweight and obesity are defined as an abnormal fat accumulation with a respective Body Mass Index (BMI) of ≥25 kg/m² and ≥30 kg/m² by the World Health Organization (WHO) (22). Obesity is an established risk factor for type 2 diabetes mellitus and its associated complications (23). The underlying metabolic dysfunction is due to chronic systemic inflammation that can lead to insulin-resistance (24). During the last three decades, epidemiological data and several cohort and case-control studies have shown an association between obesity and CRC (25-27). The simultaneously rising incidence of obesity and CRC in patients younger than 50 years of age indicates a particular contributing role of metabolic dysfunction in the development EOCRC (4). Liu et al. prospectively analyzed a patient cohort of more than 85,000 women aged 25 to 42 years, that were part of the The Nurses' Health Study II (4). An association between obesity and EOCRC was found among this patient collective. The recent analysis of Hussan et al. showed an increasing trend in CRC among young patients with obesity, that

could not be demonstrated in other types of gastrointestinal cancer (28). Focusing on the age of diagnosis in patients with CRC, an increased cancer riskwas shown after a diagnosis of type 2 diabetes, especially in men younger than 55 years (29).

A recent study on the molecular characteristics of EOCRC showed that inflammatory mechanisms, such as deregulated redox homeostasis as one of the hallmarks of CRC in young patients, play a distinct role (30). The major pathways that are involved in these mechanisms are altered nuclear factor erythroid 2-related factor 2 (NRF)-mediated oxidative stress response, glutathione metabolism, and the chemokine (C-X-C motif) ligand 12 - C-X-C motif chemokine receptor 4 (CXCL12-CXCR4) signaling axis (30). These findings suggest that metabolic dysfunction and obesity represent an important contributing factor in CRC development in young patients.

A chronic inflammatory environment is caused by the proinflammatory endocrine activity of adipose tissue, affecting energy homeostasis and glucose metabolism (31). Inflammatory macrophages can accumulate within adipose tissue in obese patients and trigger inflammation, which leads to systemic metabolic dysfunction, including insulin resistance (32). The presence of macrophages is a hallmark of proinflammatory adipose tissue. They form crown-like structures in subcutaneous and visceral fat deposits (32). Furthermore, adipose tissue-derived inflammatory mediators have been shown to induce macrophage polarization towards a proinflammatory phenotype in an in vitro model (33). In other obesity-related comorbidities, such as non-alcoholic fatty

liver disease (NFLD) or steatohepatitis (NASH), inflamed adipose tissue has been associated with activation of liver macrophages as a determinant for liver fibrosis (34). Proinflammatory macrophage polarization in tissue macrophages can provide a link between the proinflammatory systemic state in obesity and a chronic inflammatory environment in colon tissue, which in turn can trigger carcinogenic mechanisms in colon epithelium through inflammatory stress.

Itaconate is a macrophage-specific metabolite, which is produced in proinflammatory macrophages, and which is known to have tumor promoting effects (35). TAMs in tumor-bearing mice as well as monocytes isolated form patients with ovarian cancer showed increased itaconate production (35). Identifying the role of macrophage metabolism and itaconate in a chronic inflammatory state due to metabolic dysfunction and obesity could lead to innovative approaches to screening diagnosis and treatment of CRC.

b. Role of chronic inflammation in colorectal cancer

Chronic inflammation is closely linked to two systems of the human body that have major roles for survival: the immune system with the ability to fight infection and the metabolic system that can provide stored energy during a period of low nutrition (24). Immunity and metabolism are therefore in a continuous state of interplay through inflammatory pathways. Both systems share several mediators, including hormones, cytokines, transcription factors, signaling proteins and lipids. A chronic inflammatory state functions as a stressor and promotes tissue damage that can lead to neoplasia. Once a genetic mutation leads to

oncogene activation, inflammation will contribute to cell proliferation, tumor establishment, growth and metastasis. CRC is a cancer type known to be closely associated with chronic inflammation. Even though less than 2% of CRC is colitis-associated, sporadic CRC shows similar mutations in genes and signaling pathways, such as the Wnt/β-catenin pathway, K-Ras or B-Raf activation, adenomatous polyposis coli (*APC*) inactivation, transforming growth factor(TGF)-β, tumor protein p53 (P53), and the DNA mismatch repair (MMR) proteins (36-39). The pathogenesis of both CRC types differs in the histological sequence that is followed during development of neoplasia and the initiation of cancer formation.

Numerous clinical and epidemiological studies have shown that the use of aspirin or nonsteroidal anti-inflammatory drugs (NSAIDs) is associated with a reduced risk of CRC or recurrent adenomatous polyps as well as decreased CRC mortality (40-45). Furthermore, low-dose aspirin therapy seems to slow progression of a tumor that is already established. A recent cohort study of more than 300,000 patients in the United Kingdom demonstrated that new use of low dose aspirin was associated with a reduced risk of advanced stage CRC (Duke's B-D) at diagnosis (43). In 2015, the USPSTF started recommending low-dose aspirin for chemoprevention of CRC in patients with increased cardiovascular risk aged 50-59 years (46).

Independent of its pathogenesis, CRC is infiltrated by immune cells such as macrophages, neutrophils or lymphocytes, that induce and maintain cancer-related inflammation (47). In colon adenomas, the precursor lesions of sporadic

CRC, TAMs with low major histocompatibility complex class 2 (MHC II) expression were observed, and the density of these macrophages correlates with tumor progression (48). This suggests that mechanisms within the TME lead to macrophage polarization towards an anti-inflammatory phenotype during the development of cancer. Furthermore, high-grade adenomas have been shown to consist of a higher fraction of anti-inflammatory macrophages than low-grade adenomas (49). This leads to the conclusion that macrophages of an anti-inflammatory type seem to have a role in malignant transformation of colorectal adenomas towards CRC.

The link between immunity and cancer through inflammation was observed as early as the 19th century by the German pathologist Rudolf Virchow, when he described white blood cells as part of the tumor mass. In 1986, the American pathologist Harold Dvorak investigated angiogenesis within tumors, considered these mechanisms similar to those in wounds and depicted tumors as ‘wounds that do not heal’ (50). Inflammatory tissue injury causes chemotactic signaling that attracts immune cells to repair damage, and TAMs are the major cell type orchestrating the pathways within the TME, to either promote or suppress tumor development in CRC (51). These opposing functions of TAMs are characterized by a respective dominating metabolic pathway of the macrophage that can be affected by extracellular signals within the tumor environment. This polarization into different functional subsets can be affected by proinflammatory cytokines (52, 53), leading to the conclusion, that there is a

direct connection between metabolism, inflammation and macrophage differentiation affecting tumor behavior.

Itaconate is a metabolite within inflammatory macrophages, and a regulator of cellular metabolism as well. It regulates glycolysis and leads to succinate accumulation through inhibition of succinate dehydrogenase (54). This can lead to decreased production of reactive oxygen species (ROS) and altered activation of numerous transcription factors, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), hypoxia-inducible factor 1α (HIF1α), signal transducer and activator of transcription 3 (STAT3), and activator protein 1 (AP-1) (35, 54). NRF2 is a superordinate regulator of these anti-inflammatory functions, that is affected by itaconate (54). In anti-inflammatory macrophages, itaconate can further boost anti-inflammatory functions (54). Since anti-inflammatory macrophages play a role in tumor progression in CRC, this suggests that itaconate affects CRC growth.

c. Tumor-associated macrophages in colorectal cancer

The ability of macrophages to adapt to various environments and to provide a wide variety of functions in tissue is due to dynamic adjustments of their cellular metabolism. These metabolic pathways can be affected by the particular TME inducing the metabolic reprogramming, which in turn leads to different cell phenotypes.

i. Cellular metabolism and different phenotypes of tumor associated macrophages

Metabolic reprogramming can occur as a result of different stimuli on TAMs, e.g. mediators secreted by cancer cells, signals from cells within the tumor microenvironment, self-secretion or indirect stimuli such as hypoxia. Although switching between phenotypes is a continuous transition with intermediate types present, two main macrophage phenotypes have been described: an M1-subtype with primarily inflammatory functions and an M2-subtype, with predominantly anti-inflammatory and immunosuppressive activity (**Figure 1**). This simplified classification is an attempt to distinguish between subsets of macrophages that have a primarily - but not exclusively - inflammatory or anti-inflammatory function. The ‘waterfall model’ illustrates specific characteristics of TAMs during their development from a monocyte to an anti-inflammatory macrophage subtype (55). During this process, monocytes that initially present markers C-C chemokine receptor type 2 (CCR2) and lymphocyte antigen 6 complex (Ly6C), undergo functional and therefore phenotypical changes, losing Ly6C and gaining MHC II expression (55). This demonstrates the continuous transition of monocytes and macrophages with overlapping cell surface markers during all stages of development.

Depending upon their phenotype, macrophages prefer specific metabolic pathways for their energy homeostasis. The characteristic metabolic profiles of inflammatory and anti-inflammatory macrophages lead to distinct phenotypes with respect to cellular metabolism, which can be studied instead of targeting cell

surface markers (**Table 2**). While aerobic glycolysis is the main pathway in proinflammatory macrophages receiving M1 stimuli, anti-inflammatory M2 macrophages are characterized by slower rates of aerobic glycolysis and primarily fatty acid oxidation (56, 57). The classically activated inflammatory M1 macrophages show induction of glycolysis through the AKT/mTOR/HIF pathway (35). Aerobic glycolysis is an inefficient pathway with a high rate of glucose consumption, but it is essential for rapid energy production and biosynthesis. M1 macrophages utilize this pathway for host-defense against pathogens, including the production of ROS to kill bacteria or tumor cells. A slower rate of aerobic glycolysis within M2 macrophages is necessary for the production of cytokines (57). In contrast to M1 macrophages, the M2 subset macrophages show increased oxidative phosphorylation (OXPHOS) (35). As shown in hepatocellular carcinoma, cancer cells can promote glycolysis in M2 macrophages through soluble mediators, increasing the gene expression of the glycolytic enzyme 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) (58). Therefore, glycolysis plays a role in both macrophage phenotypes, but the respective energy production focuses on different glycolysis-associated pathways.

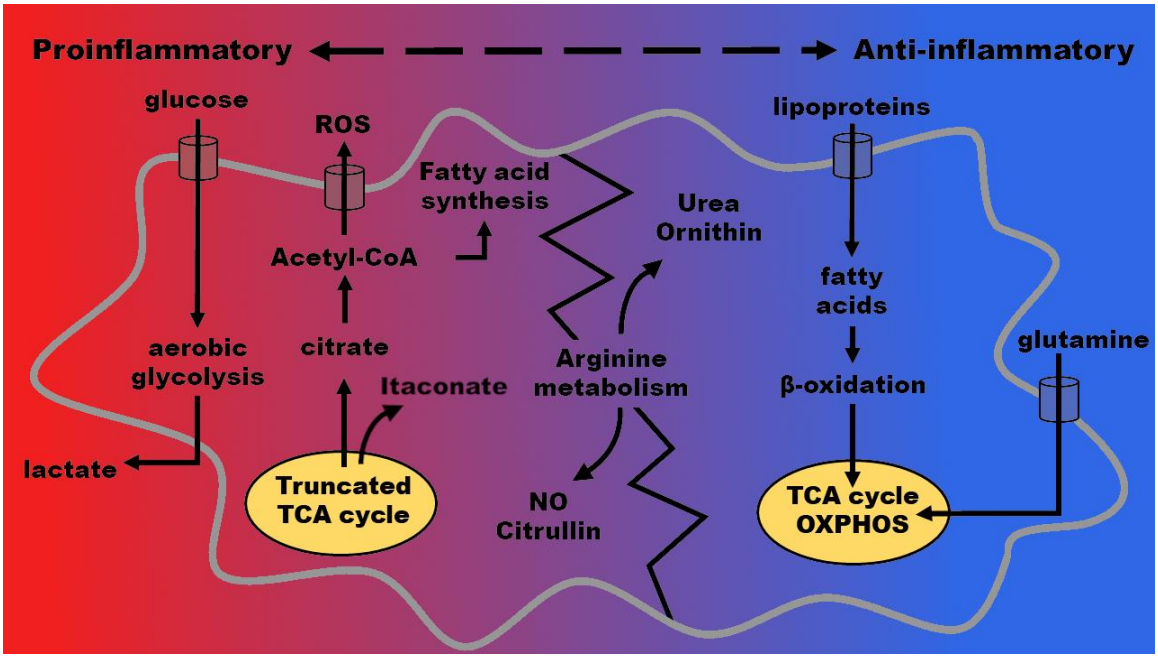

Figure 1. Metabolic pathways in proinflammatory and anti-inflammatory phenotypes of TAMs.

Simplified model showing the dominating metabolic pathways in both extremes of phenotypes in TAMs. As macrophages can switch between proinflammatory and anti-inflammatory phenotypes continuously by changing their cell metabolism, metabolic pathways can overlap between both types. Proinflammatory macrophages focus on aerobic glycolysis, truncated tricarboxylic acid cycle (TCA cycle) and fatty acid synthesis for energy homeostasis of the cell. Anti inflammatory macrophages use the TCA cycle, oxidative phosphorylation and β oxidation as their major energy sources.

ROS = reactive oxygen species; TCA cycle = tricarboxylic acid cycle.

Table 2

Human macrophage characteristics and marker expression depending on their metabolic phenotype (inflammatory versus anti-inflammatory)

Phenotype	**Proinflammatory (M1-like subtype)**	**Anti-inflammatory (M2-like subtype)**
Cell surface markers	CD11c, CD16, CD80, CD86, MHC II	CD163, CD206, CD209
Factors inducing differentiation	IFN-γ, TNF, LPS, ATP	IL-4, IL-10, IL-13, TGF-β
Metabolic pathways	aerobic glycolysis, truncated TCA cycle (Itaconate production), fatty acid synthesis	β-oxidation, oxidative TCA cycle
Secreted factors	IL-1β, IL-6, IL-8, IL-12, IL-23, IL-27, TNF-α, CXCL1, CXCL9, CXCL10, CXCL11, CCL2, CCL5, RNI, ROI, COX2	IL-10, IL-13, IL-1RA, TGF-β, CCL17, CCL18, CCL22, CCL24, Arg1, COX1, VEGF, PDGF

The listed cell surface markers, factors and metabolic pathways are not exclusively present in only one of these macrophage phenotypes. Since macrophages can switch between phenotypes showing continuous transitions, these characteristics might overlap. However, the characteristics that are shown in this table are more likely to be present in the respective phenotype.

CD = cluster of differentiation; MHC II = major histocompatibility complex class 2; IFN = interferon; TNF = tumor-necrosis factor; LPS = lipopolysaccharides; ATP = adenosine thiotriphosphate; IL = interleukin; IL-1RA = Interleukin-1 receptor antagonist; TCA cycle = tricarboxylic acid cycle; CXCL = chemokine (C-X-C

motif) ligand; TGF = transforming growth factor; CCL = CC-chemokine ligand; RNI = reactive nitrogen intermediates; ROI = reactive oxygen intermediates; COX = cyclooxygenase; Arg1 = arginase 1; VEGF = vascular endothelial growth factor; PDGF = platelet-derived growth factor.

ii. The dual role of tumor-associated macrophages in colorectal cancer

In contrast to other solid human cancers, TAMs in CRC seem to have the ability to both support and suppress tumor growth. Tumor-promoting mechanisms are known to result from an interplay between cancer cells, the tumor microenvironment and TAMs. It is hypothesized, that tumor initiation is fostered by mutagenic mechanisms from a chronic inflammatory environment in the subepithelial stroma (59). Proinflammatory M1 macrophages that produce reactive oxygen and nitrogen species, are able to potentiate this effect, triggering oncogenic mutations in the adjacent epithelial layer (**Figure 2**). Once neoplasia is initiated, the tumor recruits additional bone marrow-derived monocytes from the bloodstream and stimulates myelopoiesis by releasing growth factors and chemotactic signals such as CC-chemokine ligands 2 and 5 (CCL2, CCL5), vascular endothelial growth factor (VEGF) and TGF-β (59-61). In adipose tissue, a similar mechanism is described, where CCL2 expression leads to increased macrophage infiltration and inflammation, which in turn is associated with insulin resistance (62). Macrophage colony stimulating factor-1 (M-CSF or CSF-1) has been shown to be produced by CC cells in order to attract and ‘re-educate’ macrophages (61). During the early stages of tumor development, neoplastic

cells seem to first attract monocytes and ensure their maturation to macrophages within the TME. After their differentiation to TAMs, cancer cells take these macrophages hostage by manipulating their metabolism through multiple signaling pathways and use these TAMs to support further tumor growth and progression. Overexpression of the chemoattractant CCL2 has been associated with advanced tumor stages, metastatic disease and poor prognosis in CRC (63, 64). Furthermore, CRC cells produce lactic acid as a by-product of predominantly aerobic glycolysis (65). Proliferating cancer cells switch their metabolism towards aerobic glycolysis, which is known as the ‘Warburg effect’. Irrespective of the availability of oxygen, they metabolize glucose to lactate, which is also secreted to induce VEGF and arginase 1 (ARG1) expression in TAMs (65). VEGF expression in macrophages was shown to be upregulated by a pathway described in hypoxia, even under normoxic conditions (65). This mechanism leads to macrophage recruitment and polarization towards the tumor promoting M2 macrophage phenotype and is therefore associated with metabolic reprogramming in TAMs. Another key mechanism for the alternative activation of tissue macrophages is the peroxisome proliferator activated receptor-γ (PPARγ) pathway (66). In animal studies, the disruption of this pathway also was associated with diet-induced obesity, insulin resistance, and glucose intolerance (66). PPARγ deficiency can also lead to increased itaconate production, which suggests that itaconate acts as an alternative regulator of M2-like polarization (54).

Furthermore, STAT3 activation leads to M2 polarization of macrophages (67). This pathway can be induced by glucagon-like peptide 1 (GLP-1), a postprandially secreted hormone that improves insulin resistance (67). TAMs also promote tumor development by inducing interleukin 10 (IL-10) production in CRC cells through a STAT3 pathway (68, 69) and produce cytokines such as VEGF to induce tumor angiogenesis and tumor growth (70).

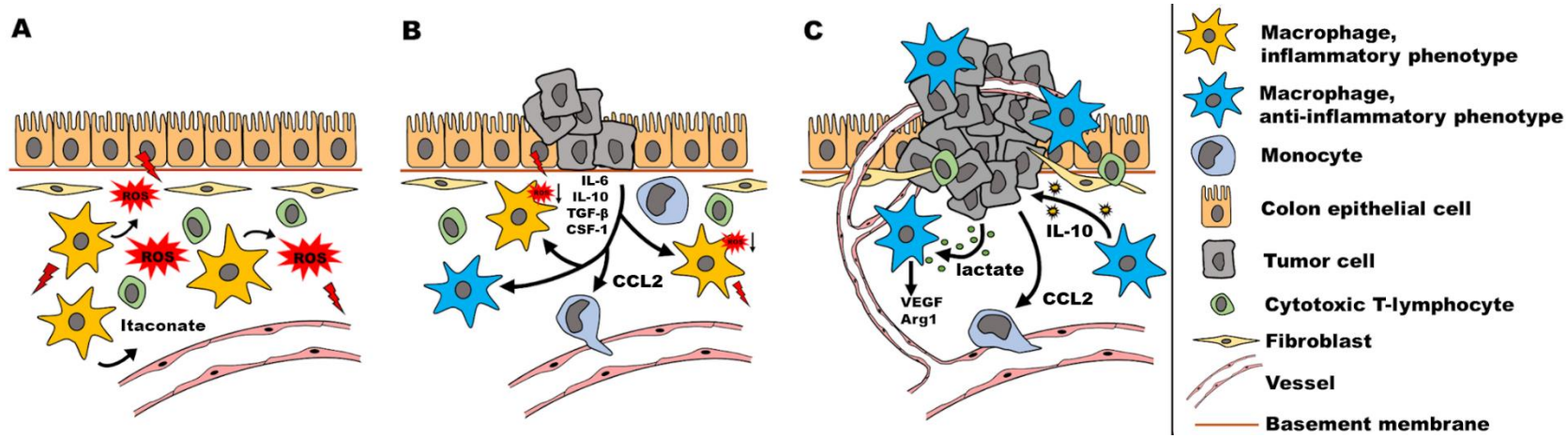

Figure 2. TAMs in chronic inflammation and CRC development.

A *Tissue-resident macrophages with a proinflammatory phenotype might be able to trigger the onset of CRC in the presence of a mutagenic activation of oncogenes in colon epithelial cells due to inflammatory stress and itaconate production.* ***B*** *During early cancer development CC cells produce chemokines (CCL2) to attract bone narrow-derived monocytes and induce macrophage differentiation releasing cytokines and growth factors such as IL-6, IL-10, TGF-β and M-CSF (CSF-1).* ***C*** *CC cells release mediators such as lactate to induce TAM polarization into an anti-inflammatory phenotype. Reprogrammed macrophages show an increased expression of vascular endothelial growth factor (VEGF) and Arginase 1 (Arg 1), promoting angiogenesis and tumor growth. Furthermore, anti-inflammatory TAMs promote tumor development by inducing IL-10 production in CC cells.*

ROS = reactive oxygen species; IL = interleukin; TGF = transforming growth factor; M-CSF = Macrophage colony-stimulating factor.

iii. Macrophage phenotypes as prognosticators in colorectal cancer

The density of recruited macrophages and their metabolic phenotype were found to be associated with different clinical outcomes in CRC patients. Despite the heterogeneity among study methods used to investigate the degree of TAM infiltration, a high TAM density within the primary tumor is associated with an improved prognosis in CRC patients (71). In other solid tumors, such as gastric, urogenital and head and neck cancers, a high TAM density is accompanied by worse overall survival (71). A higher degree of infiltrating macrophages in the invasive front of CRC, in particular those with an M1 phenotype, is associated with a better prognosis in a stage-dependent manner (72, 73). Furthermore, it is inversely correlated to lymph node and liver metastases (74, 75). While M2 macrophages seem to be more prevalent in stage II CRC, M1 macrophages are predominant in less invasive T1 tumors (76). This indicates that M1 macrophages are primarily responsible for tumor initiation because of inflammatory mechanisms increasing oncogenic potential. Further in the course of the tumor, cancer cells recruit additional bone marrow-derived blood monocytes and reprogram their metabolism to induce M2-polarization (59).

Investigating the different functions and phenotypes of TAMs during tumor development, which in turn promote and suppress tumor growth, is the basis for developing new diagnostic and therapeutic targets, especially in EOCRC. The specific role of itaconate, that can regulate macrophage polarization in tumors, is

currently unknown in CRC. **Table 3** provides an overview of current studies investigating TAM phenotypes and therefore indirectly TAM metabolism in CRC.

Table 3

Studies on CRC investigating cancer-related mechanisms related to the phenotype and metabolism of TAMs

	Year	Study Model	Aims & Objectives	Results	Conclusions Regarding TAM Metabolism
Colegio et al. (65)	2014	Murine/ murine cell line	• Identification of tumor signals that lead to functional polarization of macrophages • Lactic acid levels in cell line media • The effect of lactic acid on macrophage polarization towards an anti-inflammatory M2-like phenotype using syngeneic LLC tumors • M2-marker expression by macrophages induced by lactic acid	• Tumor cells induce VEGF & Arginase1 expression in macrophages via HIF1α • Comparison of intracellular metabolites of M1-line & M2-like macrophages revealed that lactate & pyruvate levels were most different • Lactic acid induced *FIZZ1*, *MGL1* & *MGL2* in bone-marrow derived macrophages	• Tumor cell-derived lactic acid had an important signaling role in macrophage polarization & therefore tumor growth • Lactic acid induced expression of genes that are defined as markers of M2-like anti-inflammatory macrophages (VEGF, Arginase 1, *FIZZ1*, *MGL1* & *MGL2*)
Deng et al. (77)	2010	Murine	• Effect of blocking *STAT3*-mediated signaling in macrophages in a transgenic mouse line • Tumor development in inflamed colon & cecum in *STAT3* knockout mice & the effect of microflora on these processes	• 20% of *STAT3* knockout mice showed pronounced colitis at 8 weeks of age; the rate further increased in older mice. Macrophage density increased in the colon of these mice • The inflamed colon developed visible polyps with associated carcinoma • A higher density of intestinal microflora was found in the stool of *STAT3* knockout mice compared to controls	• *STAT3* knockout promotes tissue inflammation in the colon of this transgenic mouse line, suggesting a role in macrophage polarization towards an M1-like proinflammatory subtype • Abnormal immunity in the bowel mucosa might induce changes in the microflora
Edin et al. (72)	2012	Human tissue	• Identification of macrophage phenotypes in CRC in relation to prognosis	• The amount of iNOS positive and CD163 positive	• A higher density of iNOS positive cells (M1 subtype, proinflammatory) is

			in CRC in general & in subgroups defined by microsatellite instability (MSI) screening status & CpG island methylator phenotype (CIMP)	cells both correlated inversely with tumor stage • A higher amount of iNOS positive cells associated with better prognosis, independent of MSI & CIMP status • No significant survival associations found in groups of CRC with different iNOS/CD163 ratios	accompanied by a higher amount of CD163 positive cells (M2-like subtype, anti-inflammatory) & correlated with better prognosis in a stage dependent manner
Feng et al. (78)	2019	Human tissue	• Macrophage density & proportion of CD206 positive macrophages as prognostic/predictive biomarkers in stage II CC	• A high CD206/CD68 ratio was associated with poor disease-free survival & poor overall survival • CD206/CD68 ratio had a better prognostic efficacy than density of macrophages (CD68 positive cells), or of CD206 positive macrophages or other clinicopathologic high-risk factors • Disease-free survival & overall survival were improved in patients with a high CD206/CD68 ratio receiving adjuvant chemotherapy, but not in patients with a low CD206/CD68 ratio receiving adjuvant chemotherapy	• In stage II CC, a higher proportion of CD206 positive macrophages (M2-like subtype, anti-inflammatory) in relation to the overall density of macrophages (CD206/CD68 ratio) is associated with poor disease-free & overall survival rates. • A higher proportion of anti-inflammatory M2-like macrophages (higher CD206/CD68 ratio) is associated with beneficial effects on survival in stage II CC patients that receive adjuvant chemotherapy
Herbeuval et al. (68)	2004	Human cell lines	• Interactions between macrophages & tumor cells including IL-6, IL-10 & *STAT3* activation	• Media of cultured macrophages can stimulate IL-10 production in several human colon adenocarcinoma cell lines through a mechanism involving IL-6 • Recombinant IL-6 (but not recombinant IL-10), TNF-α	• Mediators released by macrophages (with proinflammatory effects, M1-like subtype) induced *STAT3*-mediated IL-10 production by CC cells that can lead to M2-polarization of macrophages

				and IFNα stimulated IL-10 secretion in CC cell lines • IL-10 gene regulation was mediated by *STAT3*. This mechanism was regulated by IL-6	
Koelzer et al. (79)	2016	Human tissue	• Intra-tumoral & stromal macrophage density in CRC • Direct cell contact between cancer cells (tumor buds) & macrophages • Predominant macrophage phenotype in CRC	• A higher density of intraepithelial macrophages correlated with less tumor budding • A higher density of stromal macrophages correlated with larger tumor diameter & less lymph node metastasis • Frequent contact between tumor buds & macrophages was present in tumors with: higher grade, lymph node metastasis, mismatch repair deficiency & BRAF mutation as well as in patients without adjuvant therapy, • 40% of macrophages (CD68 positive) were also CD163 positive, 60% were iNOS positive • High counts of CD163 positive macrophages were associated with lower tumor grade, less lymph node metastasis, less advanced T-stage, absence of lymphatic invasion, KRAS wild type genotype & a non-significant survival benefit	• Macrophage phenotypes, classified by cell surface markers, show an association with survival. A high CD163 positive macrophage count (M2-like subtype, anti-inflammatory) was associated with a non-significant survival benefit. Proinflammatory iNOS positive macrophages (M1-like subtype) showed no association with survival
Malesci et al. (80)	2017	Human tissue/ human cell lines	• Macrophage density at the invasive front of the primary tumors & metastatic lymph nodes	• High macrophage densities in primary tumors & lymph nodes were associated with a lower risk of tumor recurrence after resection as well as with	• Proinflammatory macrophages (M1-like subtype) & 5-fluorouracil showed a synergistic effect on cancer cell death

			• Prognostic/predictive value of macrophages & neutrophils & interactions with 5-fluorouracil adjuvant therapy	better disease-free survival in 5-fluorouracil treated patients • Patients with stage III CRC & high macrophage density in tumors (particularly metastatic lymph nodes) show significantly better 5-year-disease-free survival than patients with low macrophage density • Cancer cell death was not increased by 5-fluorouracil exposure after coculturing with unpolarized macrophages • Coculturing cancer cells with M1-like macrophages nearly doubled the cell death rate. This was even further increased by exposure to 5-fluorouracil	
Nandi et al. (81)	2016	Murine	• Effects of *CCR6* knockout on growth of a syngeneic transplanted CC in mice • Macrophage density in syngeneic transplanted CC in *CCR6* deficient mice • Effect of CCL20 on macrophage accumulation in vivo • Effect of macrophage accumulation on tumor growth in *CCR6* deficient mice • Correlations between CCR6 expression with that of the macrophage marker CD163 & with CCL2, IL-1α, IL-6 & TNF-α	• Macrophage density was lower & tumor growth was delayed in *CCR6* deficient mice compared to wild type mice • Macrophage accumulation was greater in response to CCL20 than to CCL2 • Macrophage depletion led to reduced tumor growth • Higher macrophage density in wild type mice accompanied by increased expression of CCL2, IL-1, IL6, but not TNF-α, compared to *CCR6* deficient mice • Expression of *CCR6* correlated with CD163, CCL2, IL-1α & TNF-α	• Macrophages accumulating in response to CCL20 and *CCR6* interaction secrete proinflammatory factors (M1-like subtype) • Expression of *CCR6* correlates with the expression of cell surface marker CD163 (M2-like subtype, anti-inflammatory)

Oosterling et al. (82)	2006	Murine	• Comparison of mRNA expression profiles, tumor load & survival between animals with macrophage-depleted tumors & controls • Identification of macrophage phenotypes within tumor tissue	• Macrophage-depleted tumors showed higher differentiation & reduced inflammatory tumor infiltrates • Higher tumor load in the peritoneal cavity & liver observed in macrophage-depleted animals • Augmented tumor development in macrophage-depleted rats correlated with decreased survival of these animals, supporting the significance of macrophage tumoricidal effector functions. • General macrophage density higher throughout control tumors compared to macrophage-depleted tumors • CD163 positive macrophages confined to the tumor periphery in control tumors & no CD163 positive cells found in macrophage-depleted tumors	• Macrophage depletion leads to higher tumor load, reduced inflammatory tumor infiltrates & to loss of the anti-inflammatory macrophage population present in control tumors (M2-like subtype)
Pinto et al. (76)	2019	Human tissue	• Identification of macrophage phenotypes in different stages of CRC	• The amount of macrophages, especially CD163 positive macrophages, was high in stage II CRC • The amount of CD80 positive macrophages was higher in less invasive T1 tumors & is associated with lower risk of cancer recurrence • Higher macrophage density and lower CD80/CD163 ratio were	• CD163 positive macrophages (M2-like subtype, anti-inflammatory) predominated in higher tumor stages and were associated with worse overall survival • CD80 positive macrophages (M1-like, proinflammatory) were associated with lower tumor stage & lower risk of recurrence

				associated with impaired overall survival	
Umemura et al. (83)	2008	Murine/ murine cell line	• Macrophage phenotype identification in murine colon adenocarcinoma	• Tumor-infiltrating monocytes/macrophages had CCR2 positive & CX3CR1 positive inflammatory monocyte characteristics • Tumor-infiltrating monocytes/macrophages were shown to produce TGF-β1, which led to upregulation of CD206 expression	• Tumor-infiltrating monocytes/macrophages cannot be classified into M1 and M2 categories since they bear overlapping characteristics • CD206 expression (M2-like subtype, anti-inflammatory) in tumor-infiltrating monocytes/macrophages is regulated by an autocrine mechanism using TGF-β1
Zhou et al. (74)	2010	Human tissue	• Association between CD68 hotspots (small areas with infiltration of CD68 positive cells above the average level of CD68 positive cell infiltration) and other clinicopathologic parameters, potential of hepatic metastasis, & 5-year survival • Macrophage phenotypes within tumor tissue	• CD68 hotspots were prognostic for survival & were associated with the potential of hepatic metastasis & the interval between colon resection & the occurrence of hepatic metastasis • Patients with stage IIIB cancer & higher macrophage density in the invasive front of the tumor had a higher 5-year survival rate after resection • Staining for identification of macrophage phenotypes showed a large proportion of HLA-DR, IL-10 & IL-12 positive macrophages, a smaller proportion of TGF-β1 positive macrophages & absence of IL-12 positive macrophages.	• Macrophage density at the invasive front of a tumor is associated with lower potential of hepatic metastasis & worse overall survival in CC • Macrophages within tumors predominantly expressed HLA-DR (M1-like subtype, proinflammatory) & IL-10 (M2-like subtype, anti-inflammatory). Fewer cells were TGF-β1 positive (M2-like subtype, anti-inflammatory). Clear conclusions on an M1-like or M2-like overall subtype by analyzing cell surface markers could not be drawn

Legend to Table 3.

CCR6 = C-C Motif Chemokine Receptor 6; CCL = CC-chemokine ligand; CD = cluster of differentiation; IL = interleukin; TNF = tumor-necrosis factor; CRC = colorectal cancer; BRAF = B-Raf proto-oncogene; iNOS = inducible nitric oxide synthase; KRAS = Kirsten rat sarcoma viral oncogene homolog gene; HLA-DR = human leukocyte antigen-DR = MHC II = major histocompatibility complex II; TGF-β1 = Transforming growth factor beta 1; CCR = C-C Motif Chemokine Receptor; CXCL = chemokine (C-X-C motif) ligand; LLC = Lewis Lung Carcinoma; VEGF = vascular endothelial growth factor; HIF = hypoxia-inducible factor; FIZZ1 = found in inflammatory zone 1 = RELM α; MGL = macrophage galactose-type lectin-1; STAT3 = Signal transducer and activator of transcription 3.

d. Perspectives for targeting tumor-associated macrophages in clinical practice

i. Tumor-associated macrophages as diagnostic markers

TAMs have the potential to be used as diagnostic and prognostic markers in CRC and possibly as therapeutic targets. Previous studies have shown that circulating TAMs and the chemokines that they produce could serve as markers in cancer diagnosis (84-86). Current research has focused on the identification of circulating TAMs in blood samples by profiling their cell surface markers in different types of cancer as a basis for developing a noninvasive screening tool. Relevant markers are cluster of differentiation (CD) 14, CD163, CD68 or hypoxia-inducible factor 2α (HIF-2α) (84-86). A combination of analyzes of cell surface markers, cytokines secreted by TAMs and soluble factors produced by other cells within the TME could be useful to determine specific cell expression profiles in CRC. Serum levels of neutrophil elastase within the TME have been shown to play a potential role as a diagnostic biomarker in CRC (87). While serum matrix metalloproteinase-9 (MMP-9) was not considered to be an appropriate screening parameter for CRC (88), tissue inhibitor of metalloproteinase-1 (TIMP-1) seems to have a potential diagnostic value (89). Targeting related factors that are expressed by TAMs or neighboring cells within the tumor microenvironment and circulatory markers may further contribute to the overall diagnostic capacity.

ii. Tumor-associated macrophages as prognostic markers

Evidence for prognostic utility of markers in CRC, with a particular role of TAM phenotypes in different tumor stages, is currently evolving. The findings with respect to the association between specific TAM phenotypes and prognosis are inconclusive, suggesting a different role of TAMs during tumor progression. This could also be caused by the fact, that not only the total cell count of either M1 or M2 macrophages seems to be relevant for tumor progression, but also the distribution of these cells within the tumor environment (72). A low density of TAMs in general, as investigated by CD68+ cell infiltration in tumor tissue, was associated with worse outcome in patients with different stages of CRC (90). A high proportion of CD163+ macrophages was associated with lower tumor grade and less lymph node metastasis (79). Other studies report advanced tumor stages and worse prognosis positively correlating with high TAM density (76). An investigation of the prognostic effect of TAMs in patients with CRC undergoing postoperative chemotherapy recently revealed, that the CD206/CD68 ratio of TAMs can predict high risk of recurrence in patients with stage II CC (78). As adjuvant chemotherapy is not routinely recommended in these patients, identifying those patients with poor prognosis is leading to targeted and more accurate administration of chemotherapy. The presence of a high density of TAMs in primary tumor tissue and metastatic lymph nodes of stage III CRC can identify patients that benefit from 5-fluorouracil (80). In-vitro results indicating synergistic effects of TAMs and fluoropyrimidines have, however, yet to be proven in an in-vivo setting (80).

Since different TAM phenotypes are associated with tumor behavior, the metabolic reprogramming of TAMs to an ‘antitumor’ phenotype is a major aim of ongoing research. In TAMs, the NF-κB pathway is the main pathway for polarization into an antitumor phenotype. This pathway is affected by Toll-like receptors, Dectin-1 receptors and specific intercellular adhesion molecule-grabbing nonintegrin (SIGN)-related 1 receptors (91, 92). Activation of these receptors causes an adaptive immune response enhancing phagocytosis and the release of inflammatory cytokines, such as tumor-necrosis factor-α (TNF-α), IL-2, IL-10 and IL-12 (92). The yeast-derived polysaccharide β-glucan can act on these membrane receptors, thereby inducing macrophage polarization into a proinflammatory anticancer phenotype (92). Apart from NF-κB, other transcription factors can also be regulated to induce M1-like polarization or to inhibit M2 polarization in macrophages, such as interferon-regulatory factor (IRF), STAT protein, HIFα and several microRNAs (53).

Pathways that are known to be involved in macrophage activation and reprogramming in the acute immune response could also play a role in a chronic inflammatory setting, consequently affecting the onset and development of CRC. Identifying inflammatory mediators in obesity that support the polarization of tumor-promoting macrophages could not only help identify patients at high risk of CRC due to metabolic dysfunction, but also serve as a basis for targeting these mediators in patients with obesity or type 2 diabetes mellitus. The effects of obesity and its associated inflammatory stressors on macrophage polarization,

TAM metabolism and therefore tumor behavior in patients with CRC, need further elucidation.

e. Conclusions

Tumor-promoting inflammation is one of the hallmarks of cancer and TAMs are able to orchestrate these mechanisms based on their cellular metabolism. Interactions between TAMs, tumor cells and other components within the TME regulate cancer establishment, tumor growth and metastasis. CRC is closely related to chronic tissue inflammation. Metabolic dysfunction in patients with obesity has the potential to induce reprogramming in TAMs through inflammatory mechanisms. The macrophage metabolite itaconate is produced during TAM polarization and it is known to have tumor promoting effects. Investigating the role of itaconate and other metabolites in TAMs can elucidate processes specific for the onset and progression of CRC on the basis of inflammatory pathways, particularly in EOCRC. There is a potential to detect new diagnostic and prognostic targets for the improvement of neoadjuvant and/or adjuvant therapies in CRC.

CHAPTER III

INFLAMMATORY PATHWAYS LINKING OBESITY AND EARLY-ONSET COLON CANCER

a. Background

The pathogenesis of EOCRC and the role of obesity and metabolic dysfunction are poorly understood. A systemic low-grade proinflammatory state due to the dysregulation of obesity-related hormones and low dietary quality in obesity and diabetes have been postulated to increase CRC risk (93, 94). Inflammatory mechanisms are mediated by immune cells, with macrophages and their phenotypical changes playing a central role in CRC (94, 95). M1-like adipose tissue macrophages contribute to a systemic proinflammatory state in obesity, thereby increasing cancer risk. Tumors having a high proportion of anti-inflammatory M2-like TAMs in CRC are associated with poor overall survival (95). Obesity-related hormones, such as leptin and adiponectin, are secreted by white adipose tissue and affect macrophage polarization and cytokine expression (96, 97). These hormones thereby regulate inflammation, insulin resistance and energy homeostasis (98). Altered hormone levels induce phosphoinositide kinase-3 (PI3K)/AKT activation in CC, causing increased cell survival,

hyperplasia and proliferation (94, 99). Inflammatory mechanisms mediated by this pathway include cytokines such as interleukin 6 (IL-6), TNF-α or IL-1β and link obesity and inflammation to CRC carcinogenesis through TAM-mediated CRC cell progression (94, 95).

Central molecules for the control of cell proliferation, differentiation and tumorigenesis within the gastrointestinal tract include downstream targets of the PI3K/AKT pathway, such as Neurogenic locus notch homolog 4 (Notch4) and GATA binding proteins (GATA) (100).

This chapter will introduce the NOTCH4-GATA4-IRG1 axis as a link between inflammation and sporadic CRC, and will discuss this pathway as a new potential immunotherapeutic target in individuals affected with obesity and EOCRC. The genes described as part of the NOTCH4-GATA4-IRG1 axis are based on an RNA sequencing (RNA-seq) analysis using Ingenuity software, which is further described in Chapter IV (methods). Genes and their functions are listed in **Table 4**.

Table 4

Genes and their functions as part of the NOTCH4-GATA4-IRG1 axis

Gene	Encoded protein	Function	Cancer-related role of genes in macrophages	Role of genes in cancer
LEP	Leptin	• Obesity-related hormone produced by adipocytes	• Leptin induces tumor promoting anti-inflammatory IL-8 expression in macrophages; • It regulates monocyte and macrophage recruitment and promotes angiogenesis through their expression of VEGF and IL-6, which enhances tumor progression in breast cancer; • Induces T-cell apoptosis via INF-γ in immune suppressive myeloid-derived suppressor cells; • In monocytes, leptin promotes proinflammatory M1-like marker expression (IL-1β, IL-6, and TNF, and resistin)	• Increased leptin expression in CRC is associated with advanced tumor stage and poor prognosis; • Leptin acts as a potent mitogen and inhibits apoptosis in CC cells;
ADIPOQ	Adiponectin	Obesity-related hormone produced by adipocytes	• Adiponectin promotes anti-inflammatory M2-like macrophage polarization (CD206, AMAC-1); • It activates PPARα and suppresses NFκB signaling in M2-like macrophages, promoting anti-inflammatory cytokine expression;	• Adiponectin inhibits the PI3K/AKT pathway and activates the AMPK/mTOR axis, inhibiting carcinogenesis, tumor cell adhesion and migration in CRC; • Adiponectin inhibits tumor growth and survival in CRC by preventing fatty acid synthesis through AMPK;
PI3K/AKT	Phosphatidylinositol-3-kinase/Protein kinase B	Intracellular lipid kinase	• PI3Kγ induces tumor promoting cytokine expression in M1- and M2-like macrophages; • It decreases the number of TAM in CC and promotes their polarization	• The PI3K/AKT pathway promotes cell growth and survival through inhibition of proapoptotic proteins, degradation of p53 and by

			into an immunosuppressive M2-like phenotype	promoting antiapoptotic gene transcription through NFκB; • Increased activity is associated with decreased disease-free and overall survival in CRC;
NFKB	Nuclear Factor Kappa B	Transcription factor	• NFκB promotes an M2-like phenotype in macrophages with protumorigenic characteristics; • Decreases mononuclear cell infiltrates in tumors and inhibits anti-tumor cytokine expression involved in M1-like macrophage activity and Th1-skewed immune response (IL-12, CCL-2, TNF-α, IL1-β) and iNOS; • Induces M2-like protumorigenic cytokine expression (IL-10, Arginase);	• Promotes peritoneal metastasis; Inhibits TNF-α induced cancer cell apoptosis; • Induces tumor vascularization in the presence of macrophages;
SERPINE1	Plasminogen activator inhibitor-1 (PAI-1)	Serine proteinase inhibitor	• PAI-1 induces recruitment and M2-like phenotype polarization in monocytes/macrophages (IL-6, CD163); PAI-1 expression strongly correlates with M2-like CD163 expression in CC	• Elevated plasma PAI-1 levels are associated with increased risk for CRC and correlate with liver metastasis, tumor size, differentiation, serosa infiltration, Duke's stage, and lymphatic metastasis; • PAI-1 expression is associated with decreased overall survival, increased metastasis and invasion in rectal cancer;
PPARA	PPARα	Transcription factor	• PPARα inhibits NFκB-related proinflammatory gene transcription (iNOS, MMPs, TNF-α)	• Reduced expression in CC compared to normal colon tissue; • Prevents neoplastic transformation of colon epithelial cells and CC growth;
PPARG	PPARγ	Transcription factor	• PPARγ is frequently inactivated by hypermethylation in peripheral blood monocytes in patients with CRC;	• PPARγ functions as a cancer suppressor in CRC, inhibiting tumor promoting NFκB-induced gene transcription;

			• Itaconate produced by the enzyme encoded by IRG1 decreases PPARG expression in M2-like macrophages;	• It induces cancer cell apoptosis, inhibits proliferation, angiogenesis and promotes an anti-inflammatory tumor microenvironment in CRC;
IRG1	Aconitate Decarboxylase 1 (ACOD1)	Dicarboxylic acid with anti-inflammatory and carcinogenic effects	• ACOD1 is part of the citric acid cylce and typically expressed in M1-like macrophages; • IRG1 can be expressed in M2-like macrophages and is associated with tumor progression;	• IRG1 is expressed in CC, but carcinogenic mechanisms are barely understood; • It is associated with growth, invasion, tumorigenesis and advanced tumor stage in gliomas; • It is highly expressed in monocytes isolated from ovarian carcinoma patient's ascites
GATA1/2/3/4/5/6	GATA binding protein1/2/3/4/5/6	Transcription factor	• GATA2 expression is associated with an M1-like phentoype, regulating LPS-induced IL-1β expression in macrophages; • GATA3 is highly expressed in M2-like macrophages;	• GATA1 promotes cell proliferation, migration and invasion in CRC; • GATA2 is highly expressed in CRC, which is associated with an increased risk of disease recurrence and decreased disease-free survival; • GATA3 is downregulated in CRC, which is associated with poor differentiation, lymph node metastasis, increased resistance to chemotherapy and poor prognosis; • GATA4 is upregulated in CC cells adapted to an acidic microenvironment; • GATA4 and GATA5 are inactivated by hypermethylation in CRC; • GATA 6 enhances cell migration and invasion in CC

NOTCH1/2/3/4	Notch receptor1/2/3/4	Transmembrane receptors	• NOTCH1, NOTCH2 and NOTCH3 are upregulated in M1-like macrophages compared to M0 and M2-like phenotypes; • NOTCH1 promotes M1-like polarization direct metabolic reprogramming in macrophages, shifting cellular metabolism to glycolysis; • NOTCH4 promotes an anti-inflammatory macrophage phenotype by decreasing M1-like gene expression (IL-6, IL-12, CD80, CD86);	• Increased expression of NOTCH1 and its downstream gene HES1 is associated with advanced colon tumor stage and poor overall survival in CRC; • NOTCH2 expression is associated with increased overall survival in CRC; • High NOTCH3 expression is found in poorly differentiated CRC and associated with poor prognosis; • High NOTCH4 expression is associated with advanced tumor stage, decreased disease-free survival and overall survival; Increased expression was found in liver metastases compared to primary cancer tissue;
DLL4	Delta Like Canonical Notch Ligand 4	NOTCH ligand	• DLL4 is associated with an M1-like macrophage phenotype;	• DLL4 enhances tumor growth and stem cell frequency in CC; • It is associated with decreased overall survival in CRC;
HES1/HEY1	Hairy and Enhancer of split 1/Hairy and Enhancer of split-related with YRPW motif protein 1	Notch target genes	• HES1/HEY1 decrease expression of a subset of proinflammatory cytokines (CXCL1, IL-6, IL-12);	• Enhances tumorigenicity of stem-like cancer cells in CC;
MMP2	Matrix Metalloproteinase-2	Metalloproteinase	• MMP2 excerts anti-inflammatory effects in macrophages, limiting proinflammatory tissue responses; • It stimulates vascular permeability to recruit peripheral blood monocytes from vessels to the tissue;	• MMP2 expression correlates with lymph node metastasis and worse outcome in CRC;
VEGFA	Vascular Endothelial Growth Factor A	Growth factor	• VEGFA expression is induced by inflammatory stimuli, such as	• VEGFα is an essential factor in tumor growth and metastasis

			NFκB in macrophages; it mediates tumor neovascularization and growth;	in CRC; its expression levels are associated with cancer risk; • High VEGFα expression levels are associated with high-grade tumors and increasing tumor size;
ABCG5/ABCG8	ATP Binding Cassette Subfamily G Member 5/ATP Binding Cassette Subfamily G Member 8	Sterol transporter (consisting of an obligate heterodimer encoded by these two genes)	• ABCG5/ABCG8 is involved in lipid uptake and metabolism. It is expressed in macrophages and upregulated by PPARA;	• ABCG5/ABCG8 expression is associated with poor survival in node-negative CRC; • The expression levels are dependent on tumor location and decrease from colon to rectum;
TNFA	Tumor Necrosis Factor α (TNF-α)	Proinflammatory cytokine	• Induced by NFκB, TNF-α is a master regulator of proinflammatory cytokine responses and is predominantly expressed in M1-like macrophages;	• TNF-α promotes cancer cell proliferation, survival, and migration, probably through facilitating epithelial–mesenchymal transition in CC; • Serum TNF-α levels are elevated in patients with CRC compared to healthy controls and are associated with advanced tumor stage and poor survival;
IL1B	Interleukin 1β (IL-1β)	Proinflammatory cytokine	• IL-1β is associated with NFKB expression, and enhances tumor growth and angiogenesis, promoting infiltration and activation of macrophages in tumors (VEGFA, MCP-1, IL-8);	• IL-1β enhances CC cell proliferation and survival and promotes cancer progression;
IL6	Interleukin 6 (IL-6)	Cytokine with pro- and anti-inflammatory effects	• IL-6 plays a central role in obesity-related inflammation and can exert both pro- and anti-inflammatory effects; it promotes an anti-inflammatory M2-like phenotype in macrophages (Arginase, CD206);	• IL-6 promotes cancer cell proliferation and inhibits apoptosis in CRC through JAKs and STAT3; • Increased IL-6 expression is associated with advanced tumor stage and decreased survival in CRC;

Legend to Table 4.

Genes, encoded proteins, and their functions in cancer-related macrophage polarization and function and cancer.

CD = cluster of differentiation; AMAC-1 = human alternative macrophage activation-associated CC chemokine-1; AMPK = AMP-activated protein kinase; mTOR = mammalian target of rapamycin; p53 = tumor protein p53; CCL = C-C motif chemokine; iNOS = inducible nitric oxide synthase; LPS = lipopolysaccharide; CXCL = chemokine (C-X-C motif) ligand; MCP-1 = monocyte chemoattractant protein-1; STAT3 = Signal Transducer And Activator Of Transcription 3

b. Leptin, adiponectin and the PI3K/AKT pathway in colorectal cancer – an overview

Phosphoinositide 3-kinase/Protein kinase B (PI3K/AKT) signaling induces immune suppression, which further promotes inflammation and tumor progression (101) (**Table 4**). PI3Kγ is an isoform of PI3K that is highly expressed in TAM, but not in cancer cells (95). This lipid kinase has been demonstrated to be a master regulator of cancer - inhibition of its gene PI3CG stimulates Nuclear Factor kappa B (NFKB) activation, one of the key pathways for intestinal carcinogenesis mediating both proinflammatory and anti-inflammatory cytokine expression (102) (**Figure 3**). Dysregulation of NFκB is a hallmark of chronic inflammation. Investigating NFκB-related cytokine responses in cancer cells and surrounding associated cells is crucial for defining inflammatory carcinogenic mechanisms. PI3Kγ-associated anti-inflammatory gene expression predicts patient survival and is therefore an appealing immunotherapeutic target in CRC (101). Silencing PI3CG in TAM leads to death of CC cells in vitro (95).

The obesity-related hormones leptin and adiponectin both alter gene expression within this pathway, thereby affecting TAM-mediated CRC progression (103, 104) (**Table 4, Figure 3**). Both leptin and adiponectin can mediate protumor effects by activating PI3K (103, 104). Within the tumor microenvironment, PI3K activation suppresses proinflammatory M1-like polarization of TAMs, creating an anti-inflammatory, tumor promoting environment (101).

The family of Akt proteins consists of the three different serine/threonine protein kinases Akt1, Akt2 and Akt 3. These kinases regulate cellular functions

relevant in cancer progression, such as cell metabolism, proliferation and survival (105). Furthermore, Akt kinases play a crucial role in macrophage polarization, with AKT1 ablation initiating M1-like polarization and AKT2 ablation leading to a more M2-like macrophage phenotype (105). Macrophage metabolism and therefore polarization directly affects tumor progression and survival in patients with CRC. Investigating the effects of obesity-related mechanisms on PI3K/Akt signaling can provide new insights on targeting mechanisms in EOCRC and obesity among the young. Leptin activates Akt by phosphorylation, thereby affecting macrophage polarization and inducing TAM-mediated tumor progression (94, 106, 107) (**Figure 3**). Adiponectin mediates anti-tumorigenic effects directly in CC cells in vitro and reduces the growth of intestinal polyps (94, 108), while on the other hand, it can induce a more M2-like macrophage polarization potentially favoring CRC progression (109). The definitive role of obesity-related hormones in TAM-mediated CRC progression and their effects in EOCRC are yet to be fully understood.

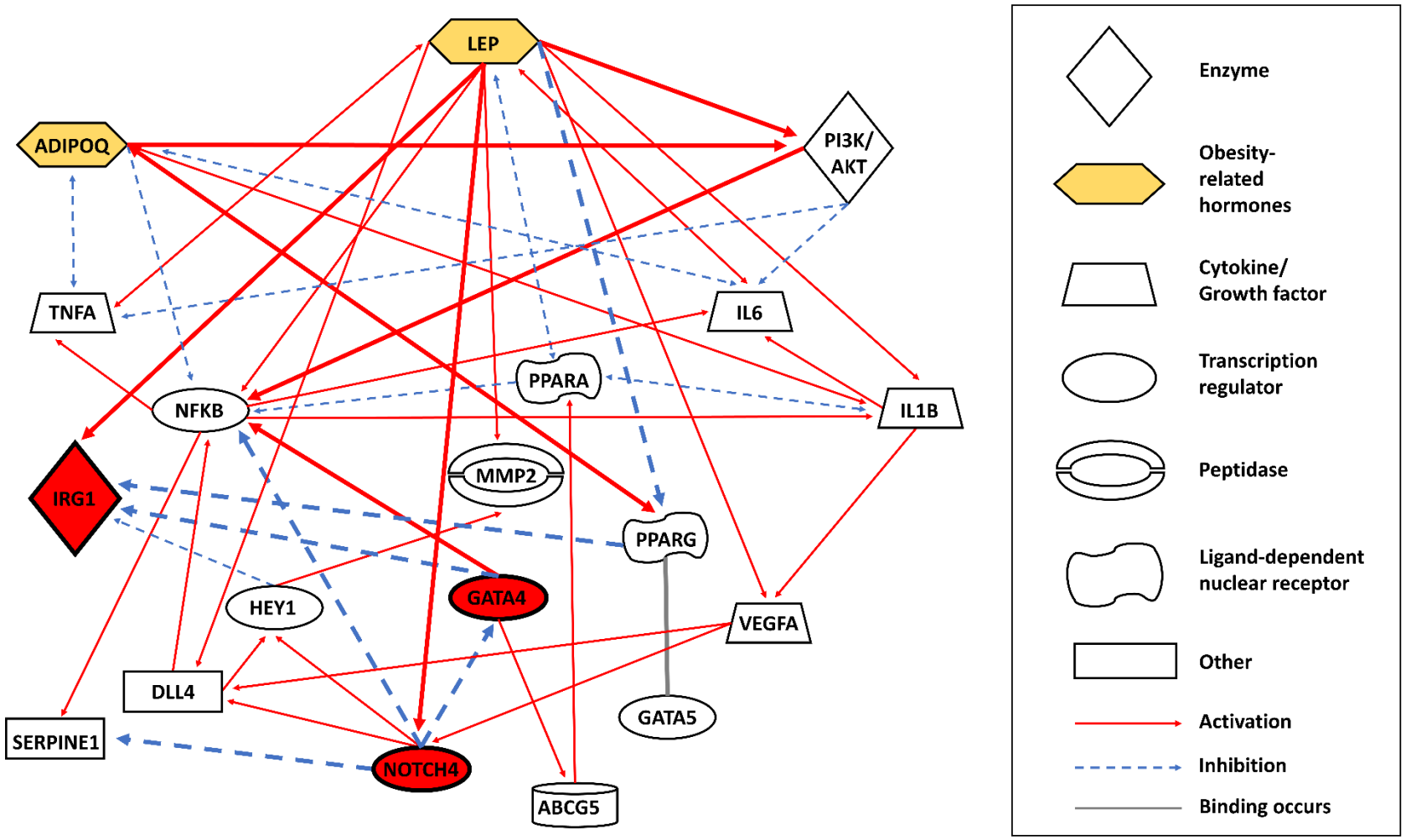

Figure 3. Structure of the NOTCH4-GATA4-IRG1 axis.

Legend to Figure 3.

Pathway network showing gene interactions of the NOTCH4-GATA4-IRG1 axis (shaded in red) and their relation to the obesity-related hormones leptin and adiponectin (yellow hexagons). Red arrows demonstrate activation, while blue dotted arrows show inhibitory effects. Major gene interactions are shown in bold.

c. GATA transcription factors – two sides of a coin

The GATA family of zinc finger transcription factors regulate gene expression during embryogenesis and the growth of several organs derived from the mesoderm or endoderm, such as the gastrointestinal tract (110, 111). During embryonic development, GATA expression is associated with cell proliferation and differentiation(110) (**Table 4**). These mechanisms have been shown to play a key role in several types of cancers, with GATA transcription factors functioning as tumor suppressors (110, 112). Recent studies, however, have shown that GATA expression is involved in carcinogenic mechanisms leading to progression of pancreatic ductal adenocarcinoma, suggesting a cancer-promoting role of the GATA protein family (113).

All GATA transcription factors bind to a canonical GATA motif in the promoters of various genes (114). GATA proteins are expressed in gastrointestinal tissue and altered gene expression is associated with several malignancies (114, 115) (**Table 4**). The function of members of the GATA family as either oncogenes or tumor suppressor genes, however, is poorly understood.

In CRC and gastric cancer, GATA4 and GATA5 have been reported to mediate tumor suppressing effects and are frequently silenced by promoter hypermethylation (116). Recent data suggests that cancer cells of solid tumors that are adapted to extracellular acidosis, however, show increased GATA4 expression (117). GATA4 enhances activity of the crucial inflammatory regulator NFκB, which in turn leads to tumor-promoting effects, such as inhibition of apoptosis (117) (**Table 4, Figure 3**).

Expression levels of GATA4 are positively associated with the proportion of proinflammatory macrophages in cancer tissue, which points towards inflammation-

driven effects in cancer development (113). In CRC, a cancer with an inflammatory etiology and macrophage-dependent development, GATA4 is expressed in around 64% of cancers (111).

GATA proteins are involved in macrophage-specific pro- and anti-inflammatory responses as part of the immune-responsive gene 1 (IRG1) pathway.

d. Itaconate – a macrophage specific metabolite promoting colon cancer

IRG1 expression is associated with several types of cancers, such as glioma, ovarian cancer and CC (18). The gene encodes for the protein aconitate decarboxylase 1, an enzyme of the tricarboxylic acid (TCA) cycle that produces itaconate from cis-aconitate (54) (**Table 4**). Itaconate mediates anti-inflammatory effects by inducing anti-inflammatory transcription factors such as NRF2 and oxidative stress reduction (118, 119). In vitro experiments commonly use the two esterified derivatives of itaconate, dimethyl itaconate (DI) and 4-octyl itaconate (OI), to investigate its effects on different types of cells in culture. While itaconate itself is polar and cannot easily cross the cell membrane, its derivatives show increased cell permeability (120). Non-specific esterases are able to release the physiological polar form of itaconate intracellularly by hydrolyzing ester groups (120).

IRG1 upregulation is associated with an M1-like macrophage phenotype, suggesting that it functions as a compensator to regulate proinflammatory cell responses (54) (**Table 4**). In anti-inflammatory M2-like macrophages, however, IRG1 upregulation can also be demonstrated under certain circumstances (96). This shows

that M2-like macrophages have the potential to contribute to an anti-inflammatory environment favoring CRC progression through itaconate.

The obesity-related hormones leptin and adiponectin can affect macrophage metabolism and polarization, thereby altering IRG1 expression (121, 122) (**Table 4, Figure 3**). The cancer promoting role of obesity-related hormones through itaconate should be clarified.

e. Notch receptors – a metabolic target in EOCRC?

The functional role of Notch signaling among different target organs and cancer types has many facets. Notch receptors are transmembrane proteins regulating tissue homeostasis (123) (**Figure 4**). Neighboring cells express transmembrane ligands that induce Notch signaling within a cell and receptor activation induces proteolytic cleavage (123). As part of these processes, the notch intracellular domain (NICD) of the receptor is released (124). After translocation of the NICD to the nucleus, it forms a transcriptional complex with transcription factor CBF1, suppressor of hairless, lag-1 (CSL) and several coactivators, modifying gene expression (124).

In CRC, Notch receptors have been shown to be involved in colon adenoma formation, and Notch expression in CRC differs among the four known Notch receptor subtypes (100, 125) (**Table 4**). Notch1 receptor expression in cancer tissue is associated with decreased overall survival in patients with CRC (126). Higher Notch1 expression levels are found in poorly differentiated CRC and in CRC with advanced tumor stage (127). The opposite role was reported for expression of NOTCH2 in CRC, showing anti-tumorigenic characteristics with increased overall survival (128). Higher

NOTCH2 expression is reported to be higher among well-differentiated tumors compared to poorly differentiated CRC, as well as in early-stage tumors compared to advanced disease (128). The third subtype NOTCH3 has oncogenic effects in CRC. Akt-dependent upregulation of NOTCH3 is associated with tumor progression and worse overall survival (129). In consensus molecular subtype 4 (CMS4) cancers, the CMS subtype of CRC with the poorest overall patient survival, NOTCH1 and NOTCH3 expression was associated with advanced tumor stage and lymph node and distant metastasis (129-131). Current data on associations of NOTCH4 expression in patient CRC tissue with clinical outcome shows inconsistent results (100, 124). Zhang et al. demonstrated that low NOTCH4 expression in cancer tissue is associated with poor differentiation, advanced tumor stage, deep wall invasion, lymph node metastasis and reduced disease-free survival (100). Furthermore, patients who are overweight and obese had a tendency to have CRC with lower NOTCH4 expression, suggesting a role of this receptor in obesity-related CRC (100). In contrast, another study by Shaik et al. showed that NOTCH3 and NOTCH4 overexpression were associated with worse overall survival (124).

In classically activated M1-like macrophages, Notch4 signaling suppresses interferon-γ (IFN-γ) and lipopolysaccharide (LPS) induced pathways leading to a more anti-inflammatory environment (132). These effects suggest Notch4 as an immunotherapeutic target in anti-inflammatory TAM-mediated CRC progression. A potential association of Notch4 with obesity has the potential to link proinflammatory mechanisms in metabolic dysfunction with anti-inflammatory, tumor promoting effects in CRC through macrophages.

Anti-inflammatory responses regulating classic activation in M1-like macrophages are an emerging subject in cancer research. Since sporadic CRC is strongly associated with dysregulation of inflammation and cellular metabolism within the tumor microenvironment, these regulatory mediators are of high importance. Currently it is assumed that anti-inflammatory mediators in M1-like macrophages function as a 'brake pedal', to control proinflammatory responses and prevent excessive oxidative stress and cell damage (54). Dysregulation of this immunomodulatory function can promote an anti-inflammatory environment facilitating cancer development.

Despite the complexity of Notch signaling, the transcription factors Hairy and Enhancer of split (HES) and Hairy and Enhancer of split-related with YRPW motif protein (HEY) have been identified as direct targets of Notch (100) (**Table 4, Figure 3**). In IFN-γ-activated macrophages, NOTCH4 diminishes proinflammatory IFN-γ-related cell responses by activating Signal transducer and Activator of Transcription 3 (STAT3) through HES1 (132). Furthermore, Notch signaling had an impact on cytokine profiles in macrophages through NFKB expression (132). Recent data revealed that NOTCH4 inhibits proinflammatory responses in macrophages, reducing cytokine expression of interleukin 6 (IL-6) and IL-12 and also M1-like cell surface receptors, such as CD80 and CD86 (132). While NOTCH1 and NOTCH3 increase NFκB activity in macrophages, NOTCH4 acts as a negative regulator of NFκB-dependent macrophage cytokine transcription (132) (**Figure 3**).

The ultimate role of NOTCH4 as either a tumor suppressor or enhancer in sporadic CRC has yet to be defined.

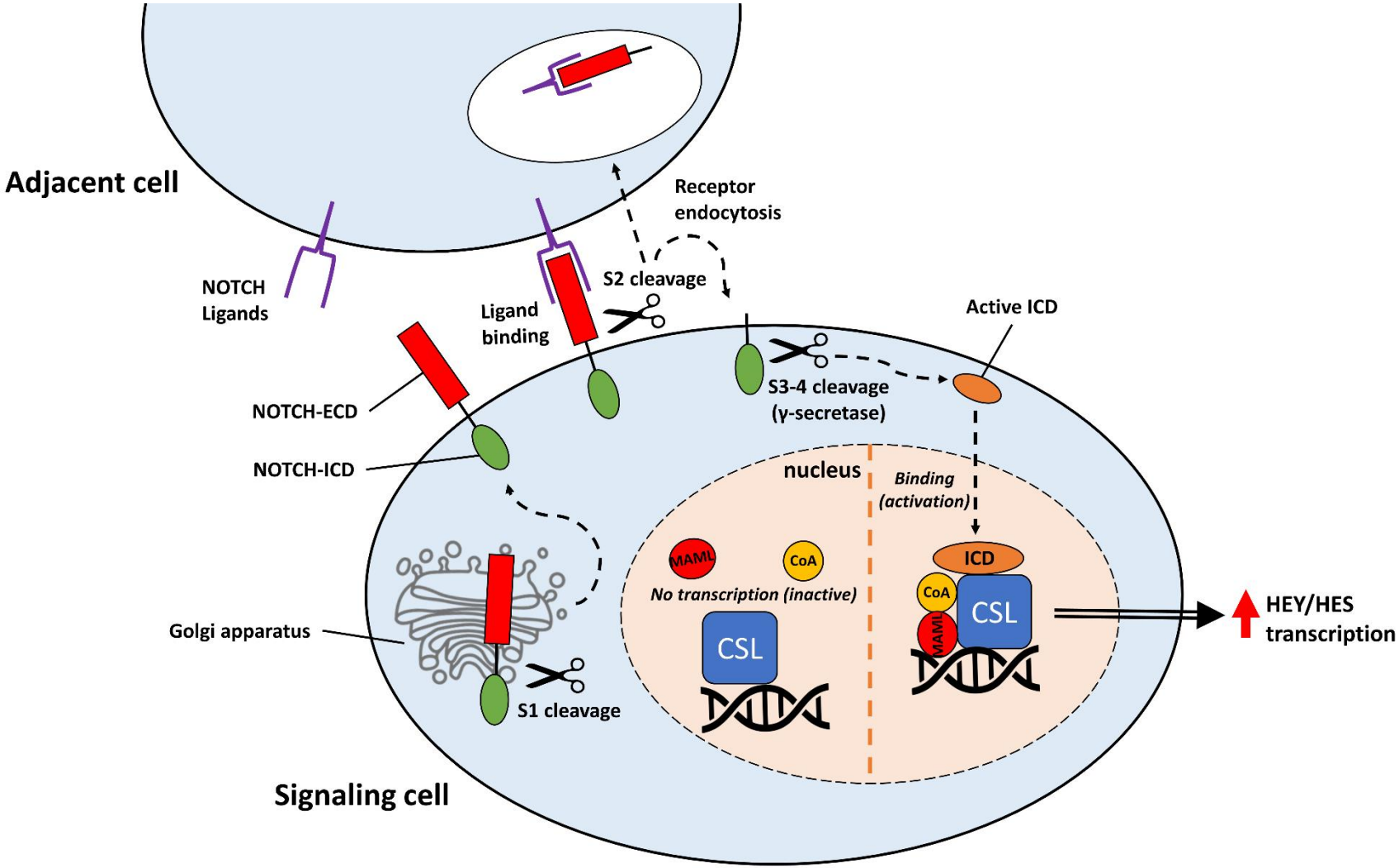

Figure 4. Cellular mechanisms of Notch signaling between cells within the TME.

Legend to Figure 4.

The Notch receptor (red and green) of a signaling cell is modified undergoing two proteolytic cleavages (S2, S3-4) after binding to its ligands on an adjacent cell (purple). The Notch intracellular domain (NICD, green) is released, transferred to the nucleus, binds to the DNA-binding CSL (CBF1, Su(H) and LAG-1) protein and induces gene transcription.

- Cleavage at Site-1 (S1): Step during Notch maturation. A furin-like convertase creates a heterodimeric Notch receptor with an extracellular domain non-covalently bound to the transmembrane/intracellular fragment.
- Cleavage at Site-2 (S2): Ligand binding of the mature Notch receptor induces a conformational change that enables a membrane bound disintegrin and metalloproteinase (*ADAM) to cleave the extracellular receptor domain. Rate-limiting step and obligatory for S3 to occur.*
- Cleavage at Site-3 and Site-4 (S3-4): Cleavage at the Notch transmembrane domain at Site-3 and Site-4 by γ-secretase. Initiating Notch signaling by releasing the intracellular domain.

NOTCH = Neurogenic locus notch homolog 4; ECD = extracellular domain; ICD = intracellular domain; MAML = mastermind-like protein 1; CoA = coenzyme A; CSL protein = CBF1, Su(H) and LAG-1 protein; HEY = Hairy and Enhancer of split-related with YRPW motif protein; HES = Hairy and Enhancer of split

f. Conclusion

Sporadic CRC is strongly associated with proinflammatory mechanisms that are chronically enhanced in patients with obesity. The increasing incidence of EOCRC and rising rates of obesity worldwide suggest a link through inflammatory mechanisms. The obesity-related hormones leptin and adiponectin mediate pro- and anti-inflammatory effects in CRC, respectively. In tumor development and progression, anti-inflammatory cytokines within the tumor microenvironment secreted by TAM play a crucial role. TAM-mediated cell responses due to leptin and adiponectin stimuli in CRC are yet to be investigated. The NOTCH4-GATA4-IRG1 axis provides a gene network that has an impact on cancer progression and patient survival through the PI3K/AKT pathway, NFκB- and PPARγ-related mechanisms. Furthermore, the NOTCH4-GATA4-IRG1 axis takes part in processes regulating tissue remodeling and EMT as critical steps for tumor progression and metastasis. Genes involved are tightly associated with proinflammatory cytokine expression, including IL-1β, IL-6 and TNF-α. Leptin and adiponectin affect these mechanisms, providing a potential link between obesity and EOCRC. The effects on the anti-inflammatory pathway signaling, involving NOTCH4, GATA4 and IRG1, however, still need to be elucidated. Anti-inflammatory mediator production in M1-like macrophages is physiological and functions as a ‘brake pedal’ to prevent excessive proinflammatory stress responses. A dysfunction of these mechanisms favoring tumor development by overly enhanced activation of the anti-inflammatory

NOTCH4-GATA4-IRG1 axis in TAM provides a new potential carcinogenic mechanism in TAM-mediated CRC progression.

Investigating obesity-related hormonal effects on TAM-mediated pro- and anti-inflammatory cytokine expression in CRC is important and may provide new immunotherapeutic targets for the treatment of TAM-mediated EOCRC.

CHAPTER IV

HYPOTHESIS, SPECIFIC AIMS AND EXPERIMENTAL PLAN

a. Key objective

To develop an in-vitro coculture model of human TAM and CC cells and to study the cellular responses to the obesity-related hormones leptin and adiponectin and the macrophage-specific carcinogenic metabolite itaconate, which may promote CC progression.

b. Hypothesis

Leptin induces metabolic reprogramming in tumor-associated M2-like macrophages towards a tumor-promoting phenotype by regulating gene expression of the NOTCH4-GATA4-IRG1 axis.

c. Specific aims

Aim 1:

Determine the effects of obesity-related hormones (leptin and adiponectin) on CC-related gene expression of anti-inflammatory tumor promoting macrophages and CC cells by establishing a cell line coculture model.

Aim 2:

Assess whether itaconate is produced in CC and determine the effects of itaconate on CC-related gene expression profiles of anti-inflammatory macrophages and CC cells.

Aim 3:

Determine age-related and obesity-related gene expression in samples of patients with CC, their relation to leptin and adiponectin and their impact on patient survival.

d. Experimental plan

The purpose of this project was to develop an in-vitro model of anti-inflammatory M2-like macrophages similar to TAM and CC cells. We anticipated that created TAM-like macrophages would show an anti-inflammatory cytokine and cancer-related gene expression profile that would mimic TAM function in human CC. This macrophage phenotype is associated with cancer progression, more advanced tumor stage and decreased overall survival in patients with sporadic CC.

After characterizing created macrophages to confirm their phenotype and after obtaining baseline expression values, we treated cells with leptin and itaconate derivatives. Since cell membranes are not permeable for itaconate, two well-established cell membrane permeable itaconate derivatives (OI and DI) were used in this model.

Pro- and anti-inflammatory gene expression as well as regulatory changes of associated transcription factors (PPARγ), which were reported to be associated with cancer progression in sporadic CC were assessed.

The results from our in-vitro studies were combined with RNA-seq data that were obtained from several databases that were either publicly available or primary sources from University studies. Age- and obesity-related genes of the NOTCH4-GATA4-IRG1 axis were identified and a gene network was created using functional annotation analysis (Ingenuity software; **Figure 3**). The effects of obesity-related hormones and itaconate on these target genes were studied in-vitro. Finally, survival analyses were performed to determine genes that are associated with decreased overall survival in patients with sporadic CC.

e. Cell culture

The human leukemia cell line THP-1 (TIB202, RRID: CVCL_0006) was purchased from the American Type Culture Collection (ATCC, Manassas, USA (catalog no.) and authenticated using short tandem repeat analysis (133). Cells were incubated in RPMI-1640 medium (ATCC, Manassas, USA) supplemented with 10% fetal bovine serum (FBS) (ATCC, Manassas, USA), 10,000 units/mL penicillin, 10 mg/mL streptomycin, 25 µg/mL amphotericin B and maintained at 37 °C with 5% CO_2. THP-1 cells were seeded at $3x10^5$ cells/ml into 24-well cell culture plates and transformed into an M2-like macrophage phenotype within 14 days. Cells were differentiated into “young M0” macrophages using 100ng/ml phorbol 12-myristate-13-acetate (PMA) (Sigma-Aldrich) for 72 hours. After 48

hours of rest in medium without PMA, the cells were polarized into an M2-like anti-inflammatory phenotype by treatment with 20ng/ml IL-4 (R&D Systems, Minnesota, USA) and 20ng/ml IL-13 (R&D Systems, Minnesota, USA) for 96 hours. In order to compare macrophages that were actively polarized into an M2-like phenotype with M0 macrophages that rested without polarization treatment, an alternative “aged M0” macrophage type was created by allowing differentiated macrophages to rest in growth medium alone for 5 days after PMA treatment rather than polarizing them with interleukins. Our protocol for creating an M2-like macrophage subtype has been published (134) (**Figure 6, Appendix**). Dose-response experiments were performed for each treatment by incubating cells with either leptin (n=20), OI (n=20) or DI (n=20) (Sigma-Aldrich, St. Louis, USA). Cellular cytokine expression after treatment with four different doses of each compound (n=5) was determined at four time points including 3, 6, 18 and 24 hours of cell treatment (96).

HT-29 CC cells were seeded at a cell density of $2x10^5$/ml/well into 24-well cell culture plates, followed by a 24-hour resting period. Cells were then treated with a dose of either leptin (n=10), adiponectin (n=10), OI (n=10) or DI (n=10) for 3, 6, 18 and 24 hours, respectively.

In coculture, THP-1-derived macrophages and HT-29 CC cells were combined for 24 hours before cell treatment with either one dose of leptin (n=10) or adiponectin (n=10).

f. Patient samples

All patients signed written informed consent. This study was approved by the University of Louisville Institutional Review Board and adheres to the requirements of the Declaration of Helsinki. Twenty consecutive colon cancer patients aged 40-81 years were included with primary surgery and curative resection. Inclusion criteria were diagnosis of sporadic colon adenocarcinoma without evidence of familial adenomatous polyposis (FAP) or Lynch-syndrome, and a minimum BMI of 18.5 kg/m^2 with no limitations on gender or age. Exclusion criteria were a history of inflammatory bowel disease, and neoadjuvant treatment for cancer including chemotherapy and/or radiation.

Paired tissue samples of CC tissue and adjacent normal colon tissue were collected from each patient. Normal colon tissue was harvested at >5cm distance from the tumor. Furthermore, 10 ml blood samples were collected immediately prior to surgery. All samples were processed for storage immediately after collection and kept at -80°C.

RNA sequencing (RNA-seq) data was obtained from The Cancer Genome Atlas (TCGA) (n=40), the European Genome-Phenome Archive (EGA) (n=69) (135, 136) and the ColoCare study from the University of Utah (n=12) (137). (EGA data *is hosted by the* European Bioinformatics Institut *(EBI) and the Centre for Genomic Regulation (CRG), under accession number EGAD00001000215.)*

g. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cells using the RNeasy purification kit (Qiagen, Maryland, USA). RNA was quantified with spectrophotometry (NanoDrop 1000, Thermo Scientific, Massachusetts, USA) and 20ng of total RNA was used to perform reverse transcription to cDNA according to the manufacturer's protocol. TaqMan Gene Expression Assays (Applied Biosystems, California, USA) and Fast Advanced Master Mix (Applied Biosystems, California, USA) were used for quantitative real-time PCR (qRT-PCR) using StepOne Real-Time PCR systems (Applied Biosystems, California, USA). Results for each target gene were normalized to 18S as the housekeeping gene and are given as mean ΔCT values.

h. Cell supernatant preparation and Enzyme-linked Immunosorbent Assays

Cell supernatants of cells in culture were collected and spun down at 1600 rpm for 7 minutes and separated from remaining cells. IL-10 and IL-8 concentrations were measured in the supernatants of THP-1 monocytes, "young M0", "aged M0" and M2-like macrophages according to the manufacturer's protocol using Human IL-10 and Human IL-8 ELISA Kits (Invitrogen, California, USA).

The enzyme ACOD1, encoded by Human Immune-Responsive Gene 1 (IRG1), was measured in colon cancer and normal colon patient tissue samples using ELISA (IRG1-ELISA Kit, MyBioSource, Inc., San Diego, CA, USA)

as per manufacturer's protocol. Protein concentrations are given per ug of total protein of the respective sample.

i. Flow cytometry

Macrophages were lifted from 24-well plates using the cold shock method by incubating cells with ice-cold PBS/5% FBS and placing them on ice for 45 minutes. Following this step, cells were mechanically detached using cell scrapers and washed with cold PBS/5% FBS.

THP-1 cells (n=5), young M0 (n=5) and M2 macrophage-like cells (n=5) were investigated. Non-specific binding of staining antibodies was inhibited by incubation with Fcγ-receptor block (BD Pharmingen, San Diego, USA) at room temperature for 10 minutes. Cells were then stained according to the manufacturer's instructions (BD Parmingen, San Diego, USA) with FITC-conjugated mouse anti-human CD14 and CD80 antibodies, with PE-conjugated mouse anti-human CD11b antibodies and with PE-Cy5-conjugated mouse anti-human CD206 antibodies and their isotype-matched IgG for 30 minutes at 4 °C. Cells were then fixed with 1% formaldehyde. 20,000 cells were acquired for each measurement.

Four-color flow cytometric analysis was performed and fluorescence quantitated using a BD FACSCalibur flow cytometer with CellQuest software (BD Biosciences, San Diego, USA). Gating of cells was carried out to exclude cell debris according to forward and side scatter. Cell viability after processing cells for flow cytometry with detachment of macrophages from cell culture plates was

determined in a representative set of samples (n=6) after incubating the cells with 7-aminoactinomycin-D (7-AAD) for 5 minutes.

j. Liquid Chromatography with tandem mass spectrometry itaconate level analysis of patient serum and colon tissue

Itaconate levels in colon cancer and normal colon patient tissue and in patient plasma samples were analyzed using liquid chromatography with tandem mass spectrometry (LC-MS/MS). An AB SCIEX API 4000TM tandem mass spectrometer (Concord, ON, Canada) was coupled to Waters Acquity UPLC® BEH C18 columns (2.1×100 mm, 1.7 µm), coupled with VanGuard T3 precolumns (2.1×5 mm, 1.7 µm) (Waters, Milford, MA, USA). The column temperature was held at 40°C, the injection volume was 5 µl, and the flow rate 0.25 mL/min. The mobile phase (buffer A) consisted of 0.1% formic acid-water and buffer B was comprised of acetonitrile. Mass spectrometry conditions were: 500°C, ion spray voltage (IS): -4500V, curtain gas (CUR): 25psi, gas 1 (GS1): 50psi, gas 2 (GS2): 60psi.

k. Statistical analysis

The descriptive statistics for ΔCt values were compared among the treatment dose groups for each respective gene by treatment and time point. The mean ΔCt values with either 95% confidence intervals or standard error of the mean (SEM) were presented (138). Significant maximum fold changes (FC) over all doses at various time points are reported.

A one-way Analysis of Variance (ANOVA) test was performed for each respective gene, treatment and time point. The p-values were reported for the comparisons between dose groups, with a treatment confirmed as significant in the case of at least 2 significant changes in gene expression at 2 consecutive doses and 2 different time points (139).

All calculations were performed using SAS System V9 statistical software (SAS Institute Inc., North Carolina, USA) (140).

i. Differential gene expression analysis

RNA-seq data from forty CC cases with matching normal tissue were downloaded from The Cancer Genome Atlas (TCGA), each with raw gene counts for 60,483 gene locations (**Table 5**) (135). Data in sixty-nine additional CC cases with matching normal tissue were obtained from the European Genome-phenome Archive (EGA) as pair-end raw sequencing files (fastq) (136, 141). The EGA sequencing files averaged 33 million reads per sample. The quality of the reads was assessed using FastQC (v.0.10.1), which indicated that no sequence trimming was necessary (142). The sequences were aligned to the human reference genome assembly (hg38) using Star (v.2.6) with an average alignment rate of 97% across the samples (143). Read counts for gene regions were obtained with HTSeq (v.0.10.0) using Gencode (v22) annotations, the same annotations used to derive gene counts for TCGA samples (144, 145). This produced raw counts for the same 60,483 gene locations. The TCGA and EGA files were merged and filtered to remove low expressed genes, resulting in

46,634 gene locations. The combined raw read counts were normalized using the relative log expression (RLE) method and input to a principal component analysis (PCA) which showed a good separation between the tumor and normal tissue samples (**Figure 5**). Differential gene expression for tumors relative to normal tissue was performed using DESeq2 which utilizes a negative binomial regression model (146). Patient number was added as a covariate to the regression model to account for sample pairing.

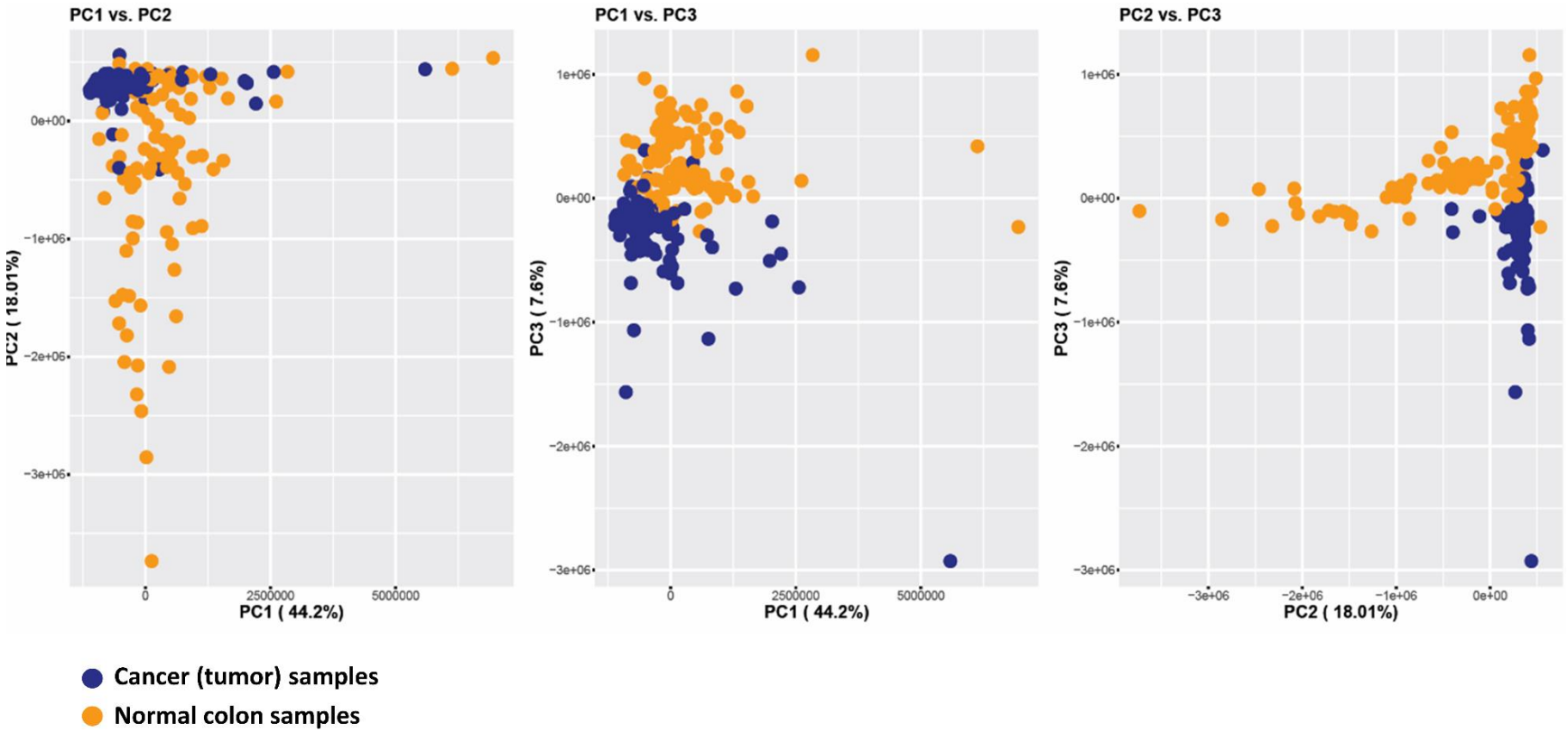

Figure 5. Principal component analysis (PCA) on tumor and normal samples.

Legend to Figure 5

*Three PCA biplots with samples plotted in two dimensions according to their projections onto principal component 1 and 2 (**A**), principal component 1 and 3 (**B**) and principal component 2 and 3 (**C**).*

*Normal colon tissue samples are shown in orange, cancer (tumor) colon tissue samples are shown in blue. The percent variance associated with each particular principal component is shown in parentheses on the x- and y-axes. 7.6% of the variability across samples (PC3) is largely due to differences in tissue type, separating tumor and normal colon samples (**B, C**).*

ii. Patient survival analysis

There was information regarding patient survival for one hundred eighty-five colon tumor samples retrieved from TCGA (**Table 5**). Twelve additional tumor samples with survival information were available from the University of Utah (ColoCare study) as paired-end raw sequencing files (fastq).

The Utah sequencing files averaged 28 million reads per sample. FastQC (v.0.10.1) indicated reads were of good quality and no trimming was necessary. The sequences were aligned to the human reference assembly (hg38) using the Star (v.2.6) aligner with an average alignment rate of 93%. Read counts for gene regions were obtained with HTSeq (v.0.10.0) using Gencode (v22) annotations. The TCGA and Utah samples were merged and filtered to exclude low expressed genes, and raw read counts normalized using the RLE method. Kaplan-Meier curves and significance levels were generated using the survival package (v3.2.7) in R to determine whether there was an association between IRG1 expression and patient survival (147, 148).

Prism 9 (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analysis, and data are shown as mean ± standard deviation or mean and 95% confidence intervals.

A McNemar analysis of binominal gene expression data of patient samples was performed. Paired samples were compared using the Wilcoxon signed rank test, and unpaired samples compared using the Mann-Whitney U test with a significance level set at 5% (2-tailed).

Furthermore, a Spearman correlation analysis was performed and graphs were created using Microsoft Excel Version 2107.

RNA-seq data analyses for differential gene expression and survival analyses were conducted using R (R Foundation for Statistical Computing, Vienna, Austria) (148).

Table 5

Sample sizes (n) for RNA sequencing data and respective analysis (differential gene expression and survival analysis)

Dataset	**RNA-seq data** **Differential gene expression** (paired samples)	**RNA-seq data** **Survival** (metadata on survival available)
TCGA	40	185
EGA	69	-
ColoCare study	-	12
Total	**109**	**197**

TCGA = The Cancer Genome Atlas; EGA = European Genome-Phenome Archive

CHAPTER V

A CELL CULTURE MODEL OF M2-MACROPHAGE MARKER EXPRESSION IN COLON CANCER

INTRODUCTION

Macrophages have various roles in the control of infections, wounds and cancer development, including inflammation, proliferation and tissue remodeling (149, 150). Tissue macrophages induce responses that can have either local or systemic effects, ranging from the regulation of wound healing to the induction of acute phase protein synthesis through hepatocytes, thereby affecting systemic inflammation and metabolism (150, 151).

Macrophages are divided into two major subtypes depending on their origin. Contrary to the erroneous assumption that tissue macrophages mostly originate from circulating bone marrow monocytes that invade tissues, most local macrophage populations are derived from embryonic progenitors that are seeded before birth (149, 152, 153). These fetal macrophages initially evolve from the yolk sac blood islands that develop during the first two weeks of gestation and in adults they are believed to maintain their population by self-renewal (154). Whether they can actually persist through adulthood and to which extent they replenish their populations is currently still discussed (155).

In contrast, bone marrow derived tissue macrophages are part of the well-established mononuclear phagocytic system (MPS) and evolve from circulating peripheral monocytes (152). Among the mononuclear phagocytic lineage, macrophages represent the final cell type after differentiation of monocytes that originate in the bone marrow (152). The monocyte-like THP-1 cell line was derived from peripheral monocytes of a one-year-old infant with acute monocytic leukemia and has been widely used as an in vitro model for demonstrating mechanisms in human monocytes and macrophages (133, 156). THP-1 cells can be differentiated into macrophage-like cells and then polarized into either proinflammatory M1 or anti-inflammatory M2 phenotypes. Due to their high plasticity macrophages can continuously switch between a predominantly proinflammatory or mostly anti-inflammatory state (157). Numerous methods have been described for differentiating THP-1 monocytes and for polarizing them into either M1 or M2 macrophages (158-162). Depending on the desired outcome of a study, different reagents are used to induce differentiation and polarization (160, 161, 163). While differentiation of primary monocytes is induced using granulocyte macrophage colony-stimulating factor (GM-CSF) or macrophage colony-stimulating factor (M-CSF), THP-1 cells require protein kinase C activators, such as PMA or byrostatin for differentiation into macrophages (164). IL-4 or a combination of IL-4 and IL-13, TGF-β1 or IL-10 can be used for polarization of THP-1 monocyte-derived macrophages (162, 165). Comparison of studies using a variety of methods, however, is problematic due to the lack of

standardized and established procedures, that use well-defined macrophage subtypes.

Macrophages are characterized by their high plasticity, changing their cellular metabolism to switch between M1- and M2-like phenotypes (166). This dichotomous model cannot fully describe their characteristics, since macrophages, specifically TAM, do not exclusively express either M1- or M2-like markers. Macrophage characterization based on this paradigm is, however, widely-used and the fundamental basis for further functional analyses (167, 168). Proinflammatory M1-like markers, such as CD80, TNF-α, CXCL10, tend to be associated with an anti-tumor immune response, while macrophages expressing other proinflammatory cytokines (IL-1β, IL-6) can mediate tumor cell migration and cancer progression (169, 170). Predominantly anti-inflammatory markers, such as CD206, IL-10, IL-8, CCL8, and CCL22 are known to be associated with advanced tumor stage and poor survival (171).

Furthermore, TAM express tumor-promoting mediators that are involved in tumor neovascularization, such as matrix metalloproteinases (MMP) and ADAM metallopeptidase with thrombospondin type 1 motif 1 (ADAMTS1) (172, 173). AP-1 is closely related to cell proliferation differentiation and apoptosis and mediates proinflammatory cytokine expression in macrophages (174). Alpha/beta-hydrolase domain containing 5 (ABHD5), a key enzyme in lipolysis regulating tumor biology (175) and toll like receptor 4 (TLR4), induced by fatty acids and mediating NFκB pathways, both also play an important role in TAM-mediated tumor progression (176). Another cancer-related gene involved in lipid

metabolism and macrophage activation is monoacylglycerol lipase (MGLL) contributing to lipid accumulation in macrophages and regulating tumor progression (176).

In the tumor microenvironment, TAM predominately have anti-inflammatory M2-like attributes that are particularly associated with tumor progression and poor overall patient survival (18, 59, 177). In order to investigate the mechanisms mediated by anti-inflammatory TAMs in a cell culture model, a distinct M2-like macrophage subtype is required. Therefore, in vitro inflammatory stimuli must be overcome, including the mechanical stress to the cells as a consequence of media changes, and/or cell treatment with proinflammatory compounds, such as PMA to induce monocyte differentiation (178, 179).

The aim is to create and characterize THP-1 monocyte-derived macrophages after differentiation and polarization into a distinct M2-like subtype within 14 days. In order to compare macrophages that were actively polarized into an M2-like phenotype with M0 macrophages that rested without polarization treatment, an alternative “aged M0” macrophage type was created by allowing differentiated macrophages to rest in growth medium alone for 5 days after PMA treatment rather than polarizing them with interleukins. An overview of the created cell types that were compared to one another is shown in **Figure 6**. Herein cytokine and tumor-associated marker gene expression, protein secretion and cell surface marker expression of cells were investigated using quantitative real-time polymerase chain reaction (qRT-PCR), enzyme-linked immunoassay (ELISA)

and flow cytometry. Detailed methods are described in Chapter IV and **Appendix**.

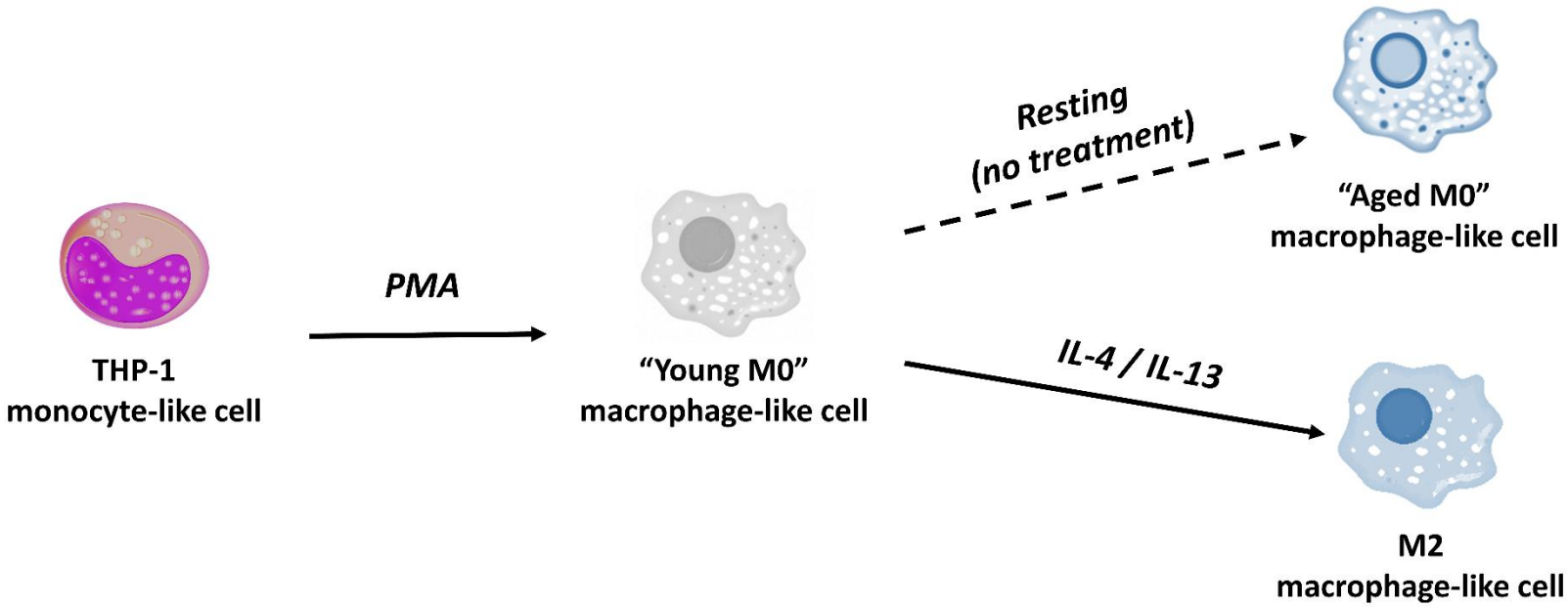

Figure 6. Model for differentiation and polarization of THP-1 monocytes into a distinct M2-like macrophage phenotype.

THP-1 monocytes are incubated in growth medium with PMA ("young M0" macrophage). Differentiated macrophages are then polarized using IL-4 and IL-13 (M2 macrophage).
Alternatively, young M0 macrophage-like cells were incubated in growth medium alone ("aged M0" macrophages).

PMA = phorbol 12-myristate-13-acetate; IL = interleukin

RESULTS

i. Expression of pro- and anti-inflammatory markers in THP-1 monocytes, M0 and M2 macrophages

Cells were differentiated into “young M0” macrophages using 100ng/ml PMA (Sigma-Aldrich) for 72 hours. After 48 hours of rest in medium without PMA, the cells were polarized into an M2-like anti-inflammatory phenotype by treatment with 20ng/ml IL-4 (R&D Systems, Minnesota, USA) and 20ng/ml IL-13 (R&D Systems, Minnesota, USA) for 96 hours. In order to compare macrophages that were actively polarized into an M2-like phenotype with M0 macrophages that rested without polarization treatment, an alternative “aged M0” macrophage type was created by allowing differentiated macrophages to rest in growth medium alone for 5 days after PMA treatment rather than polarizing them with interleukins.

Pro- and anti-inflammatory marker expression was significantly different among all cell types for CD80, TNF-α, IL-1β, CXCL10, CD206, IL-10, IL-8/CXCL8, CCL18, CCL22; each with p=<0.001, and for IL-6 p=0.033 (**Table 6 and 7**). The p-values of pairwise comparisons are provided in **Table 8**. Expression patterns (mean ΔCT values) of all markers for the respective cell types are shown as a heat map in **Figure 7**.

Table 6

Expression patterns (ΔCT values) of proinflammatory markers among cell types

Marker	THP-1 (ΔCT) N=16	Young M0 (ΔCT) N=32	Aged M0 (ΔCT) N=40	M2 (ΔCT) N=80	p-value	Significant pairwise differences
CD80	26.27 ± 1.55	24.36 ±3.37	22.87 ± 0.58	20.79 ± 0.54	**<.001**	All
TNF-α	17.88 ± 1.47	18.53 ± 0.91	18.57 ± 0.45	17.97 ± 0.73	**<.001**	THP-1 – young M0; THP-1 – Aged M0; Young M0 – M2; Aged M0 – M2
IL-1β	21.96 ± 0.51	14.78 ± 3.54	20.18 ± 0.72	21.84 ± 0.55	**<.001**	THP-1 – Young M0 THP-1 – Aged M0 Young M0 – Aged M0 Young M0 – M2 Aged M0 – M2
IL-6	28.59 ± 1.22	27.26 ± 1.74	26.92 ± 6.41	26.04 ± 1.53	**0.033**	THP-1 – M2;
CXCL10	25.75 ± 1.24	21.20 ± 0.89	20.21 ± 1.52	22.34 ± 0.76	**<.001**	All

Marker expression was investigated using quantitative real-time PCR. Results are shown as mean ΔCT values and standard deviations (SD) for all cell types. N-numbers represent the number of wells of the respective cell type created. To compare all groups, a one-way ANOVA model with a post hoc Benjamini-Hochburg correction was used. Significant results of pairwise comparisons between cell types are listed.

CD = cluster of differentiation; TNF-α = tumor necrosis factor α; IL = interleukin; CXCL = C-X-C Motif Chemokine Ligand; CCL = C-C Motif Chemokine Ligand

Table 7

Expression patterns (ΔCT values) of anti-inflammatory markers among cell types

Marker	THP-1 (ΔCT) N=16	Young M0 (ΔCT) N=32	Aged M0 (ΔCT) N=40	M2 (ΔCT) N=80	p-value	Significant pairwise differences
CD206	28.15 ± 1.14	23.99 ± 0.71	19.73 ±0.50	18.80 ± 0.47	**<.001**	All
IL-10	28.22 ± 1.55	22.88 ± 0.67	19.38 ± 0.44	18.70 ± 0.47	**<.001**	All
IL-8	19.41 ± 1.39	14.93 ± 2.21	20.69 ± 0.82	19.45 ± 0.43	**<.001**	THP-1 – Young M0; THP-1 – Aged M0; Young M0 – Aged M0; Young M0 – M2; Aged M0 – M2
CCL18	28.95 ± 0.91	28.06 ± 0.95	23.80 ± 0.42	17.23 ± 0.34	**<.001**	All
CCL22	17.96 ± 0.55	19.45 ± 1.10	19.03 ± 0.46	19.03 ± 0.40	**<.001**	THP-1 – Young M0; THP-1 – Aged M0; THP-1 – M2; Young M0 – Aged M0; Young M0 – M2;

Legend to Table 7

Marker expression was investigated using quantitative real-time PCR. Results are shown as mean ΔCT values and standard deviations (SD) for all cell types.
N-numbers represent the number of wells of the respective cell type created.
To compare all groups, a one-way ANOVA model with a post hoc Benjamini-Hochburg correction was used.
Significant results of pairwise comparisons between cell types are listed.

CD = cluster of differentiation; IL = interleukin; CXCL = C-X-C Motif Chemokine Ligand; CCL = C-C Motif Chemokine Ligand

Table 8

Corrected p-values for all pairwise comparisons of pro- and anti-inflammatory gene expression between cell Types

Marker	THP-1 vs Young M0	THP-1 vs Old M0	THP-1 vs M2	Young M0 vs Old M0	Young M0 vs M2	Old M0 vs M2
CD80	0.000	0.000	0.000	0.000	0.000	0.000
TNF-α	0.016	0.015	**0.851**	**0.879**	0.004	0.004
IL-1β	0.000	0.000	**0.796**	0.000	0.000	0.000
IL-6	**0.245**	**0.201**	0.042	**0.679**	**0.201**	**0.245**
CXCL10	0.000	0.000	0.000	0.000	0.000	0.000
CD206	0.000	0.000	0.000	0.000	0.000	0.000
IL-10	0.000	0.000	0.000	0.000	0.000	0.000
IL-8	0.000	0.001	**0.923**	0.000	0.000	0.000
CCL18	0.000	0.000	0.000	0.000	0.000	0.000
CCL22	0.000	0.000	0.000	0.005	0.002	**0.990**

p<0.05 indicates a significant pairwise comparison (paired t-test). Non-significant comparisons are bold.

CD = cluster of differentiation; TNF-α = tumor necrosis factor α; IL = interleukin; CXCL = C-X-C Motif Chemokine Ligand; CCL = C-C Motif Chemokine Ligand

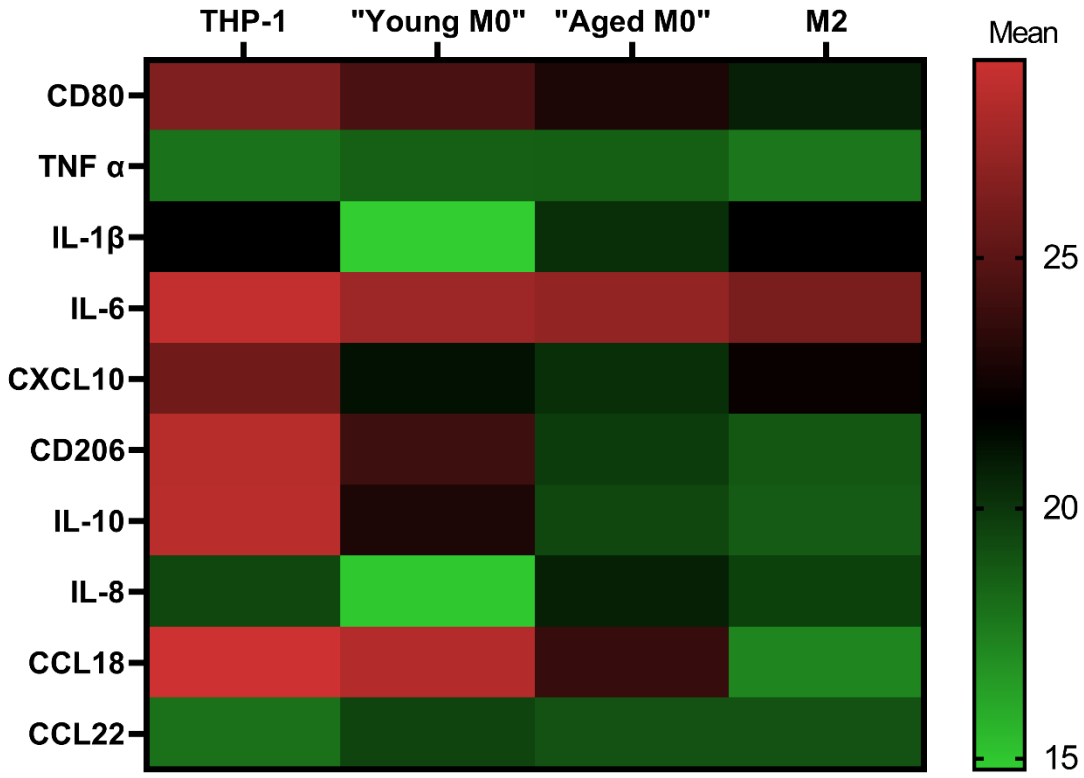

Figure 7. Marker gene expression using qRT-PCR among cell types.

Heat map of marker gene expression (ΔCT values) of pro- and anti-inflammatory markers among all cell types. Lowest gene expression is depicted by a red color, highest expression by green.

Proinflammatory macrophage markers: CD80, TNFα, IL-1ß, IL-6, CXCL10.
Anti-inflammatory macrophage markers: CD206, IL-10, IL-8, CCL18, CCL22
CD = cluster of differentiation; TNF-α = tumor necrosis factor α; IL = interleukin; CXCL = C-X-C Motif Chemokine Ligand; CCL = C-C Motif Chemokine Ligand

1. M1-associated marker expression

Overall p-values with means and standard deviations of PCR ΔCT values for all cell types are shown in **Table 6**.

Pairwise comparisons between groups demonstrated a significant difference of M1-associated CD80 between cell types due to upregulation of CD80 in “young M0”, “aged M0” and M2 macrophages compared to THP-1 monocytes (**Figure 7, Table 6**). The pro-inflammatory marker TNF-α was significantly downregulated in “young M0” and “old M0” compared to M2 macrophages and compared to THP-1 monocytes. The difference between M2-macrophages and THP-1 cells at baseline was not statistically significant (**Figure 7, Table 6**). The M1-associated marker IL-1β was significantly upregulated in “young M0” and “aged M0” macrophages compared to THP-1 cells as well as compared to M2 subtype macrophages. “Young M0” macrophages showed the highest level of IL-1β expression (**Figure 7, Table 6**). IL-6 was significantly upregulated in “young M0”, “aged M0” and M2 macrophages compared to THP-1 cells (**Figure 7, Table 6**). CXCL10 showed a significantly higher expression in “young M0”, “aged M0” and M2 macrophages compared to THP-1 monocytes. The highest level of CXCL10 expression was demonstrated in both M0 cell groups (**Figure 7, Table 6**).

2. M2-associated marker expression

Means, standard deviations and overall p-values of PCR ΔCT values for all cell types are shown in **Table 7**.

CD206 was most highly expressed in M2 macrophages, with “young M0”, “aged M0” and M2 cells showing significant upregulation compared to THP-1 monocytes (**Figure 7, Table 7**). The M2-associated marker IL-10 showed the same expression pattern as CD206, with the highest expression in M2 macrophages. “Aged M0” macrophages showed significantly higher CD206 expression compared to “young M0” macrophages or THP-1 cells, (**Figure 7, Table 7**). IL-8/CXCL8 was significantly upregulated in “young M0” macrophages compared to “aged M0”, M2, and THP-1 cells. The highest M2-associated CCL18 expression was seen in M2 macrophages with significant upregulation compared to all other cell types. “Aged M0” cells showed a significantly higher expression compared to “young M0” and THP-1 cells (**Figure 7, Table 7**). CCL22 was significantly downregulated in all cell types compared to THP-1 cells. (**Figure 7, Table 7**).

ii. Expression of tumor-associated markers in THP-1 monocytes, M0 and M2 macrophages

Tumor-associated marker expression was significantly different among all cell types for MMP2, MMP7, MMP9, MMP12, ABHD5, ADAMTS1, AP-1, MGLL each with p=<0.001 (**Table 9**). TLR4 expression showed no significant difference among cell types. The corresponding p-values of pairwise comparisons are shown in **Table 10**.

All investigated tumor-promoting MMPs were significantly increased in M2-like macrophages, with MMP2 showing the lowest upregulation in M2-like

macrophages compared to THP-1 monocytes (**Table 9, Table 10**). The metallopeptidase ADAMTS1 was significantly downregulated in M2-like, “young M0” and “aged M0” macrophages compared to THP-1 cells (**Table 9, Table 10**). ABHD5, a lipolytic factor either potentiating tumor growth or functioning as a tumor suppressor in certain types of cancer, was significantly downregulated in M2-like macrophages compared to all other cell types (**Table 9, Table 10**). Upregulation of MGLL, a key enzyme in lipid metabolism that also has both tumor promoting and suppressing effects, was most evident in both M0 phenotypes compared to THP-1 monocytes, but also significant in M2-like macrophages (**Table 9, Table 10**).

Expression of the immune-response receptor TLR4 did not differ between cell types. The transcription factor AP-1, which exerts a dual role among different types of cancers, showed slight upregulation in “young M0” macrophages compared to THP-1 cells, but no difference was found comparing M2-like macrophages versus THP-1 monocytes (**Table 9, Table 10**).

Table 9

Expression patterns (ΔCT values) of tumor-associated markers among cell types

Marker	THP-1 (ΔCT) N=16	Young M0 (ΔCT) N=32	Aged M0 (ΔCT) N=40	M2 (ΔCT) N=80	p-value	Significant pairwise differences
MMP2	19.59 ± 0.27	19.13 ± 1.10	16.72 ± 0.36	17.87 ± 0.34	**<.001**	All
MMP7	28.33 ± 3.21	18.29 ± 0.68	25.30 ± 0.92	22.92 ± 0.61	**<.001**	All
MMP9	16.55 ± 0.63	10.02 ± 0.48	11.15 ± 0.38	10.59 ± 0.42	**<.001**	All
MMP12	27.31 ± 1.80	19.62 ± 2.41	22.80 ± 0.66	19.04 ± 0.64	**<.001**	THP-1 – Young M0; THP-1 – Aged M0 THP-1 – M2; Young M0 – Aged M0; Aged M0 – M2;
ADAMTS1	19.39 ± 0.32	20.40 ± 1.54	20.29 ± 0.71	21.61 ± 1.13	**<.001**	THP-1 – Young M0; THP-1 – Aged M0; THP-1 – M2; Young M0 – M2; Aged M0 – M2;
ABHD5	18.47 ± 0.32	18.29 ± 0.63	18.78 ± 1.03	19.68 ± 1.54	**<.001**	THP-1 – M2; Young M0 – M2; Aged M0 – M2;
MGLL	21.96 ± 0.46	16.74 ± 1.51	16.94 ± 0.46	18.25 ± 0.37	**<.001**	THP-1 – Young M0; THP-1 – Aged M0; THP-1 – M2; Young M0 – M2; Aged M0 – M2;
TLR4	20.24 ± 0.25	18.27 ± 0.71	19.18 ± 3.43	18.77 ± 2.50	**0.056**	-
AP-1	25.80 ± 1.20	25.05 ± 2.90	27.00 ± 0.89	26.25 ± 1.48	**<.001**	THP-1 – Young M0; Young M0 – M2; Aged M0 – M2;

Legend to Table 9.

Marker expression was investigated using quantitative real-time PCR. Results are shown as mean ΔCT values and standard deviations (SD) for all cell types. N-numbers represent the number of wells of the respective cell type created. To compare all groups, a one-way ANOVA model with a post hoc Benjamini-Hochburg correction was used. Significant results of pairwise comparisons between cell types are listed.

MMP = matrix metalloproteinase; ADAMTS1 = ADAM Metallopeptidase With Thrombospondin Type 1 Motif 1; ABHD5 = alpha/beta-hydrolase domain containing 5; MGLL = monoglyceride lipase; TLR4 = toll like receptor 4; AP-1 = activator protein 1

Table 10

Corrected p-values for all pairwise comparisons of tumor-associated gene expression between cell types

Marker	THP-1 vs Young M0	THP-1 vs Old M0	THP-1 vs M2	Young M0 vs Old M0	Young M0 vs M2	Old M0 vs M2
ABHD5	**0.624**	**0.448**	0.001	**0.124**	0.000	0.001
ADAMTS1	0.004	0.006	0.000	**0.662**	0.000	0.000
AP-1	0.046	**0.335**	**0.120**	**0.120**	0.000	0.002
MGLL	0.000	0.000	0.000	**0.268**	0.000	0.000
MMP2	0.013	0.000	0.000	0.000	0.000	0.000
MMP7	0.000	0.000	0.000	0.000	0.000	0.000
MMP9	0.000	0.000	0.000	0.000	0.000	0.000
MMP12	0.000	0.000	0.000	0.000	0.061	0.000
TLR4	0.052	**0.210**	**0.103**	**0.210**	**0.418**	**0.418**

p<0.05 indicates a significant pairwise comparison (paired t-test). Non-significant comparisons are bold.

ABHD5 = alpha-beta hydrolase domain-containing 5; ADAMTS1 = ADAM Metallopeptidase With Thrombospondin Type 1 Motif 1; AP-1 = activator protein 1; MGLL = monoacylglycerol lipase; MMP = matrix metalloproteinase; TLR4 = toll like receptor 4

iii. Anti-inflammatory interleukin-8 and interleukin-10 protein expression in M0 macrophages and M2 macrophages compared to THP-1 monocytes

Protein expression of the anti-inflammatory cytokine IL-8/CXCL8 was significantly different among cell types (p=<0.001) (**Figure 8A**). IL-8/CXCL8 protein expression did not significantly differ between THP-1 cells, “aged M0” cells and M2 macrophages. “Young M0” cells showed a variable, but significantly increased IL-8/CXCL8 protein expression (19989.56 [95% CI 12930.32, 27048.80] pg/ml) compared to the other cell types.

Protein expression of the M2-like cytokine IL-10 differed significantly among cell types (p=<0.001) (**Figure 8B**). IL-10 was not detectable in THP-1 cells. There was no significant IL-10 protein expression in “young M0” macrophages. IL-10 protein was significantly expressed in “aged M0” (15.43 [95% CI 9.82, 20.98] pg/ml) and in M2 macrophages (13.29 [95% CI 8.75, 17.82] pg/ml), with no significant difference between these two cell types.

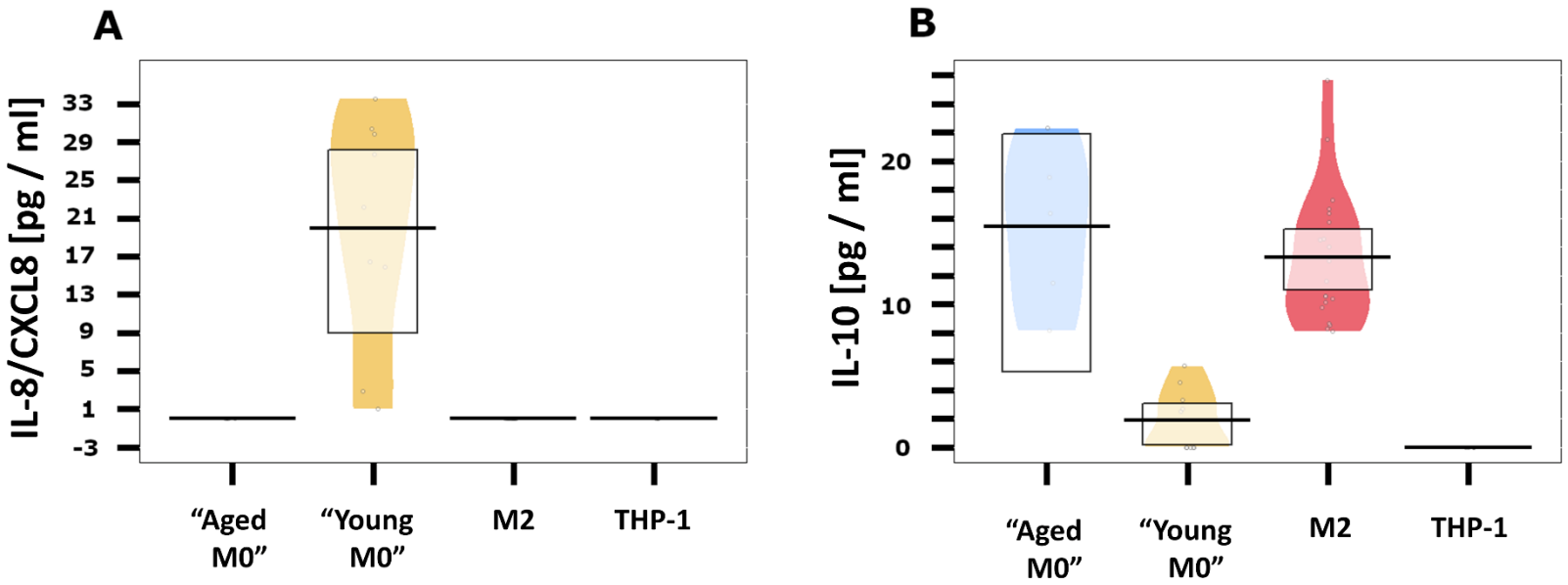

Figure 8. Pirate Plots of protein expression of anti-inflammatory markers among cell types (**A**) interleukin 8 (IL-8), (**B**) interleukin 10 (IL-10).

*IL = interleukin**;** CXCL = C-X-C motif chemokine ligand*

iv. Pro- and anti-inflammatory cell surface markers

Cell viability of differentiated macrophages after incubating cells with 7-AAD was >95% throughout all samples.

The myeloid marker CD14 was expressed in 2.06%±0.46% of THP-1 cells. "Young M0" macrophages showed low CD14 expression (3.87%±1.81% of cells). There was, however, a distinct upregulation of CD14, which was demonstrated in 69.18%±3.48% of M2 macrophages (**Figure 9**).

THP-1 monocytes exhibited low CD11b expression (4.39%±1.36% of cells). A significant upregulation of CD11b could be demonstrated in M2 macrophages (76.37%±4.68%) (**Figure 9**), with a lower expression in "young M0" macrophages (11.47%±2.65%).

The M1-like marker CD80 was not markedly expressed in any cell type (0.11%±0.07% of THP-1 cells; 0.36%±0.17% of M0 cells and 0.05%±0.01% of M2 macrophage-like cells) (**Figure 9**).

Expression of the M2-like marker CD206 was low among THP-1 cells (0.22%±0.07%) and "young M0" macrophages (0.98%±0.64%), with a significant upregulation in M2 macrophages (58.08%±2.74%) (**Figure 9**).

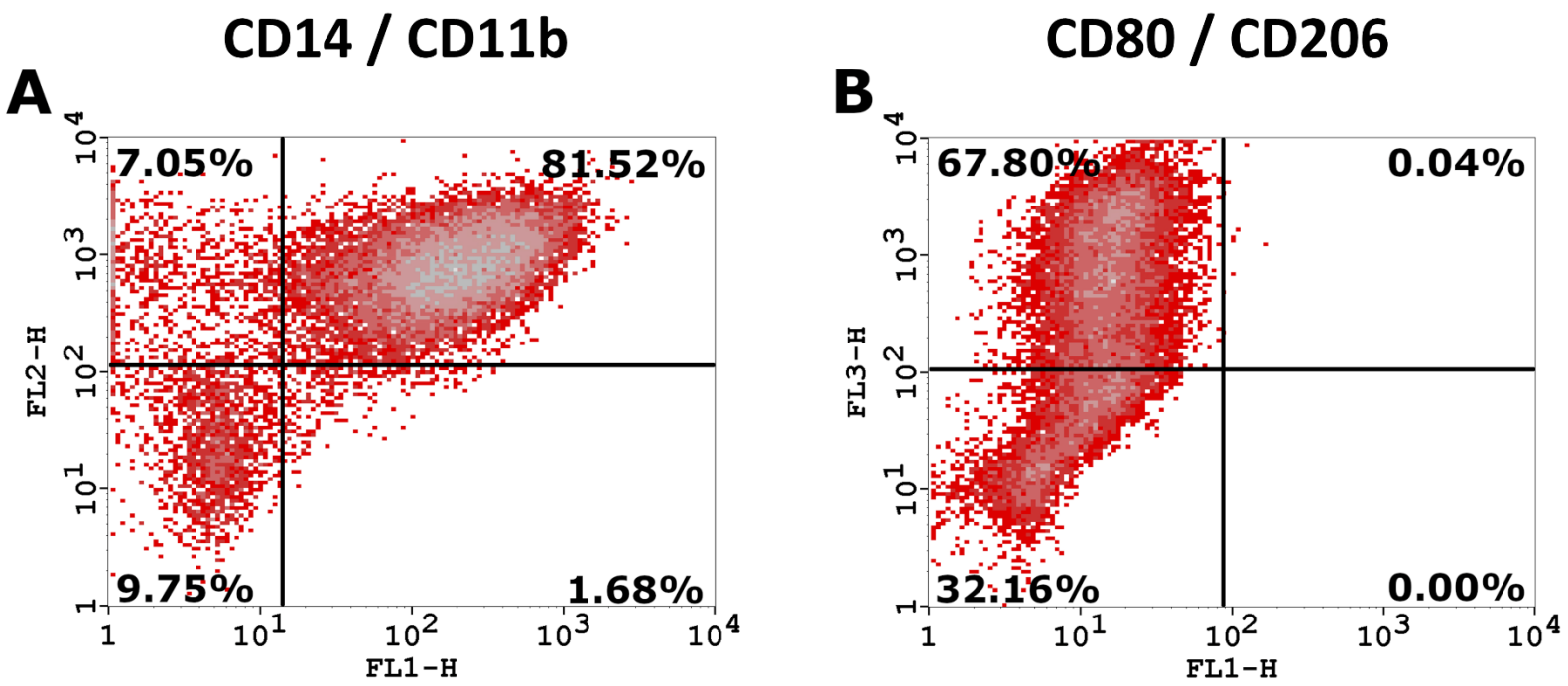

Figure 9. Flow cytometry fluorescence analysis for surface marker expression in M2 macrophages.

*Representative density plots of cell surface marker expression of (**A**) the myeloid markers CD14 (FITC, FL1-H) and CD11b (PE, FL2-H) and of (**B**) the proinflammatory marker CD80 (FITC, FL1-H) and the anti-inflammatory marker CD206 (PE-tandem conjugate, FL3-H) in M2 macrophage-like cells after the 14-day cell treatment. Total percentages of cells are shown in each quadrant.*

CD = cluster of differentiation; FITC = Fluorescein isothiocyanate; PE = phycoerythrin; FL = fluorescence channel

DISCUSSION

Macrophages mediate central mechanisms in wound healing, fibrosis and cancer growth. Specifically, the proportion of anti-inflammatory M2-like macrophages is associated with advanced tumor stage and decreased overall survival in certain types of cancers (18, 180, 181). Currently there is no distinct M2-like macrophage subtype based on an established reproducible protocol, that makes studies investigating functional analyses in cancer research comparable. Various techniques for differentiating and polarizing macrophages are available, but experimental models are rarely characterized and lack reproducibility (158-162). We have provided a protocol of a cell line model for differentiating and polarizing THP-1 monocytes within 14 days and herein characterize the distinct M2-like macrophage subtype that is thereby created (182). This provides the basis for investigating mechanisms mediated by anti-inflammatory macrophages in vitro.

One of the limitations of this study is the use of a human cell line model rather than primary cells as a basis to investigate human macrophage mechanisms in vivo. In contrast, primary macrophages are characterized by great variation in marker expression patterns and have different functional roles according to their source of origin (183). Previous data showed that PMA-treated THP-1 macrophages did not completely mimic the complexity of primary MDM activation (184). The use of a cell line, however, can provide a reproducible and standardized model to recreate functional responses in vitro. Furthermore, characteristics of THP-1 derived cells used for mimicking macrophage

mechanisms in vivo can be altered to achieve similar marker profiles to primary macrophages. Another limitation is the complexity of myeloid cell characterization due to cell plasticity and the wide variety of functional abilities of these cells. This leads to limited characterization of M2 macrophage-like cells created by this protocol. The cell treatment scheme of this study, however, demonstrates the reproducible creation of a distinct M2-like phenotype of macrophages, that can be further characterized depending on the intended experimental setting (134). Cytokine mRNA and protein expression and flow cytometry used in this study demonstrated a clear anti-inflammatory shift of differentiated macrophages and differences between resting cells versus cells receiving polarization treatment. Further analyses concerning pro- and anti-inflammatory cytokine expression, protein production, cell surface marker expression as well as functional analyses of these M2-like macrophages should follow on the basis of the underlying treatment protocol.

The differentiation of THP-1 monocytes is widely performed by incubating cells with the proinflammatory compound PMA (160, 178, 179, 185). This method of differentiation is especially suitable for mimicking primary human monocyte-derived macrophages (MDM), showing similar characteristics concerning cell morphology, macrophage surface markers and cytokine profiles (186). Pro-inflammatory responses of the macrophages that are created by this approach must decrease in order to obtain non-polarized M0 macrophages and to assure an adequate M2-like polarization process. For this reason, cells are reported to rest in medium only after PMA treatment for various periods of time (158, 161,

185). This model is based on a five-day rest period after PMA incubation, creating macrophages that mimic primary human MDM in terms of cytoplasmatic volume and cell surface adherence (185, 187).

The cytokine profile of young M0 macrophages that are described in this study demonstrated significantly increased expression of the proinflammatory markers CD80, IL-1β, IL-6 and CXCL10 compared to THP-1 monocytes, while no difference in TNFα expression was shown. Even though the term “M0 macrophages” defines differentiated macrophages in a resting state, characteristics of cellular activation (M1-like state) are usually identified in the real-world setting (188). Therefore, the standardized model presented herein clearly describes cytokine expression as a baseline threshold for future experiments. The anti-inflammatory marker IL-8, however, is most highly expressed in young M0 macrophages, and significantly upregulated compared to all the other cell types. This was confirmed by protein expression analysis, showing high variability of IL-8/CXCL8 concentrations among “young M0” macrophages. Variation in expression levels of IL-8/CXCL8 is a previously described characteristic of this cytokine in several different cell types, such as pulmonary epithelial cells or cervical cancer cells (189). THP-1-derived macrophages have also been reported to show highly variable IL-8/CXCL8 protein expression after treatment with proinflammatory stimuli (187, 189). This observation is partially understood to result from the functional role of IL-8/CXCL8, that involves synchronization of several regulatory pathways, including

NFκ-B, c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38 MAPK) (187).

The M2-like macrophages created by this model demonstrate a significant upregulation of the anti-inflammatory markers CD206, IL-10 and CCL18 compared to both “young M0” and “aged M0” macrophage-like cells, with CCL18 upregulation showing the greatest difference in M2-like cells as compared to either type of M0 cells. This demonstrates that the cell line model of this study is mimicking primary human MDM, as CCL18 is also abundantly produced by these cells following M2-like polarization (190). This cytokine induces macrophage maturation into an M2-like phenotype itself and is known to be produced by TAM in several different types of cancers (191-193). Both groups of M0 macrophages did not differ from M2-like cells with respect to CCL22 expression. Production of IL-10 in M2-like macrophages was confirmed by a significantly increased IL-10 protein expression in these cells compared to that observed in THP-1 monocytes and young M0 macrophages. IL-10 production in “aged M0” macrophages did not differ from M2 macrophages on the protein level, however, the variability in IL-10 production was high among “aged M0” cells. IL-10 is reported to be upregulated in polarized primary human MDM as well, mostly as part of an autocrine mechanism using the Signal Transducer And Activator Of Transcription 3 (STAT3) pathway (194).

Flow cytometry analysis for cell surface expression markers revealed that the distinct M2-like subtype that is created by this protocol not only shows high expression of the anti-inflammatory marker CD206, but also of both myeloid

markers CD14 and CD11b. The macrophage differentiation marker CD14 is also highly expressed by primary human MDM as a response to IL-10 and distinguishes THP-1-derived macrophages from THP-1 cells that are differentiated into a dendritic cell type with low CD14 expression (195, 196). Co-expression of the myeloid CD11b has been reported in primary human muscle tissue macrophages (197). Furthermore, the anti-inflammatory M2-like markers CD206 and IL-10 are expressed by resident macrophages of the colon lamina propria (198).

The tumor-associated attributes of created macrophages were assessed focusing on marker genes that play a role in neovascularization as well as cellular metabolism and therefore macrophage polarization.

MMPs are important cancer-related targets promoting vascularization and tumor development (172, 173). All investigated subtypes of MMPs were upregulated in the M2-like macrophage phenotype. The metallopeptidase ADAMTS1 was significantly downregulated in all cell types compared to THP-1 cells and showed the most evident decrease in M2-like macrophages. Expression of this protumorigenic gene in M2-like macrophages, however, could be demonstrated. This suggests that ADAMTS1-related pathways can be investigated using the distinct M2-like macrophage phentotype presented herein.In general, many markers regulating tumor development can be expressed in macrophages and have a dual role depending on the type of cancer they have been investigated in – such a ABHD5, MGLL, TLR4 or AP-1 (199-202). All of these markers are expressed in the M2-like macrophages created by

this model with AP-1 showing the lowest level of gene expression in M2-like macrophages. This suggests that changes in gene expression due to cell treatment or cell signaling can be assessed. The demonstrated tumor-associated gene expression patterns are the basis for expression changes in different experimental settings.

A clear similarity of cells created by this model to primary human macrophages and tumor-associated characteristics of the M2-like phenotype are to be confirmed by further functional experiments comparing THP-1 derived macrophages versus primary cells.

CHAPTER VI

THE ROLE OF ITACONATE IN COLON CANCER PATIENTS

INTRODUCTION

Metabolic overload and chronic inflammation in obese patients leads to increased oxidative stress and altered cellular metabolism, both of which are associated with colorectal carcinogenesis (203-205). These mechanisms are regulated by itaconate, an anti-inflammatory metabolite of the citric acid cycle, which is specifically produced in macrophages (54). The dicarboxylic acid itaconate is produced by the mitochondrial matrix enzyme cis-aconitate decarboxylase 1 (ACOD1). Recent studies demonstrated that itaconate mediates tumor promoting mechanisms in several types of cancer, such as melanoma, ovarian cancer, and glioma (35, 206, 207). The role of itaconate in human CC, however, is currently still unknown (18).

Cancer cells must adapt their cellular metabolism to proliferate rapidly. Metabolic reprogramming is a hallmark of cancer and various metabolic gene expression subtypes in CC have been identified. A CC subtype that exhibits downregulation of lipid metabolic gene expression patterns is associated with decreased patient survival (208, 209). Lipid metabolism also plays a key role in the functional subtypes of TAMs that, in turn, regulate inflammation and oxidative

stress within the tumor microenvironment (210). Macrophage function is characterized by cell polarization in a dynamic setting, determining either a more proinflammatory M1-like or an anti-inflammatory M2-like phenotype (211). Both functional macrophage subtypes use lipid and mitochondrial oxidative metabolism as their source of energy to a variable extent, and itaconate is typically produced in classically activated M1-like macrophages (54). Itaconate production has, however, also been demonstrated in the more anti-inflammatory M2-like subtype, as part of M2-like macrophage activation via glutamine metabolism (212). In patients with obesity, a chronic proinflammatory state due to metabolic dysfunction may cause reprogramming of the cellular metabolism in tissue-resident macrophages and TAMs, leading to cancer onset and promoting tumor growth (18).

Within the tumor microenvironment in CC, M2-like macrophages provide an anti-inflammatory environment and thereby promote cancer progression. A higher M2/M1-like ratio within the TAM population is associated with advanced tumor stage and poor patient survival in CC (72, 213). Whether anti-inflammatory itaconate released by TAMs contributes to this tumor environment, cancer onset or tumor progression in CC and the association with metabolic dysfunction in patients with obesity is poorly understood. The aim is to evaluate IRG1 gene expression, the role of the enzyme ACOD1, and the dicarboxylic acid itaconate in patients with sporadic colon adenocarcinoma using qRT-PCR, ELISA and mass spectrometry (LC-MS/MS). Detailed methods are described in Chapter IV.

RESULTS

i. Patient demographics

Clinical patient characteristics are shown in **Table 11**.

Eleven out of 20 patients (55%) were men. The mean age at diagnosis was 65.9 ± 14.9 years, with five patients (20%) diagnosed with EOCC at ≤50 years of age. Mean BMI was 29.0 kg/m^2 (range 21.6 – 40.4 kg/m^2); with eight patients (40%) normal weight, three patients (15%) overweight, and nine patients (45%) obese according to the World Health Organization (WHO) classification (214). Four of 20 patients (20%) presented with cancers with regional or distant metastases, classified as stage III or IV according to the American Joint Committee on Cancer (*AJCC*) classification.

Table 11

Clinical characteristics of patients (n=20) providing paired CC and normal colon samples for qRT-PCR, ELISA and mass spectrometry

	Total Group n=20	**≤50 years of age** n=5	**>50 years of age** n=15
Gender [% men]	55	40	60
Age [years] (mean ± SD[a])	65.9 ± 14.9	45.6 ± 4.2	72.6 ± 9.9
Tumor stage[b] [N]	- I (7) - II (9) - III (3) - IV (1)	- I (2) - II (3)	- I (5) - II (6) - III (3) - IV (1)
Mean body mass index (BMI)[c] [kg/m^2] (mean ± SD[a])	29.0 ± 6.3	32.9 ± 7.7	27.8 ± 5.5
Normal weight [N]	8	1	7
Overweight [N]	3	1	2
Obesity class 1 [N]	5	1	4
Obesity class 2 [N]	2	0	2
Obesity class 3 [N]	2	2	0

[a] *Standard deviation*

[b] *American Joint Committee on Cancer (*AJCC*) staging*

[c] *Body mass index [kg/m^2] according to class of obesity (WHO classification) (214):*

Normal weight: BMI 18.5-24.9

Overweight: BMI 25-30

Obesity class 1: BMI 30-35

Obesity class 2: BMI 35-40

Obesity class 3: BMI >40

ii. IRG1 Gene expression, ACOD1 protein analysis and itaconate levels

The role of itaconate in patient samples was analyzed on three different levels, including IRG1 gene expression, ACOD1 protein expression and by measuring the dicarboxylic acid itaconate (**Figure 10**).

The mean delta CT for IRG1 gene expression in CC tissue samples was 27.4 [95% CI (26.0, 28.7)]. IRG1 gene expression was demonstrated in 65% of CC samples, while only 5% of normal colon samples expressed IRG1 with a ΔCT of 20.4 (chi-square: 10.083, p=0.002).

On the protein expression level, ACOD1 in CC tissue was significantly higher compared to normal colon (11.8 ng/ug [95% CI (3.4, 20.2 ng/ug)] versus 3.7 ng/ug [95% CI (0.7, 6.7 ng/ug)]; p=0.002). In young patients, the mean ACOD1 protein concentration was higher in cancer and normal colon compared to old patients, however, this did not reach statistical significance (**Table 12**).

The presence of the macrophage metabolite itaconate could be demonstrated in both CC and normal colon samples. CC tissue samples showed a higher mean itaconate level compared to normal colon tissue with a high variability of levels in both tissue types and no observed significant difference (216.1 [95% CI (107.8, 324.4)] versus 153.2 [95% CI (103.4, 203.0)]; p=0.294) (**Table 12**). In young patients (≤50 years of age); however, tissue itaconate levels in normal colon were significantly higher compared to old patients (231.2 [95% CI (124.4, 338.1)] versus 125.3 [95% CI 69.6, 181.1)]; p=0.026) (**Table 12**).

The distribution of itaconate levels in paired colon tissue and plasma samples is shown in **Figure 11**. Plasma levels did not correlate with itaconate

tissue levels either in cancer samples (r_s= -0.20; p=0.414) or normal colon samples (r_s= 0.19; p=0.433). There was, however, a significant positive correlation between overall plasma itaconate levels and BMI (r_s= 0.51; p=0.021) (**Figure 12**).

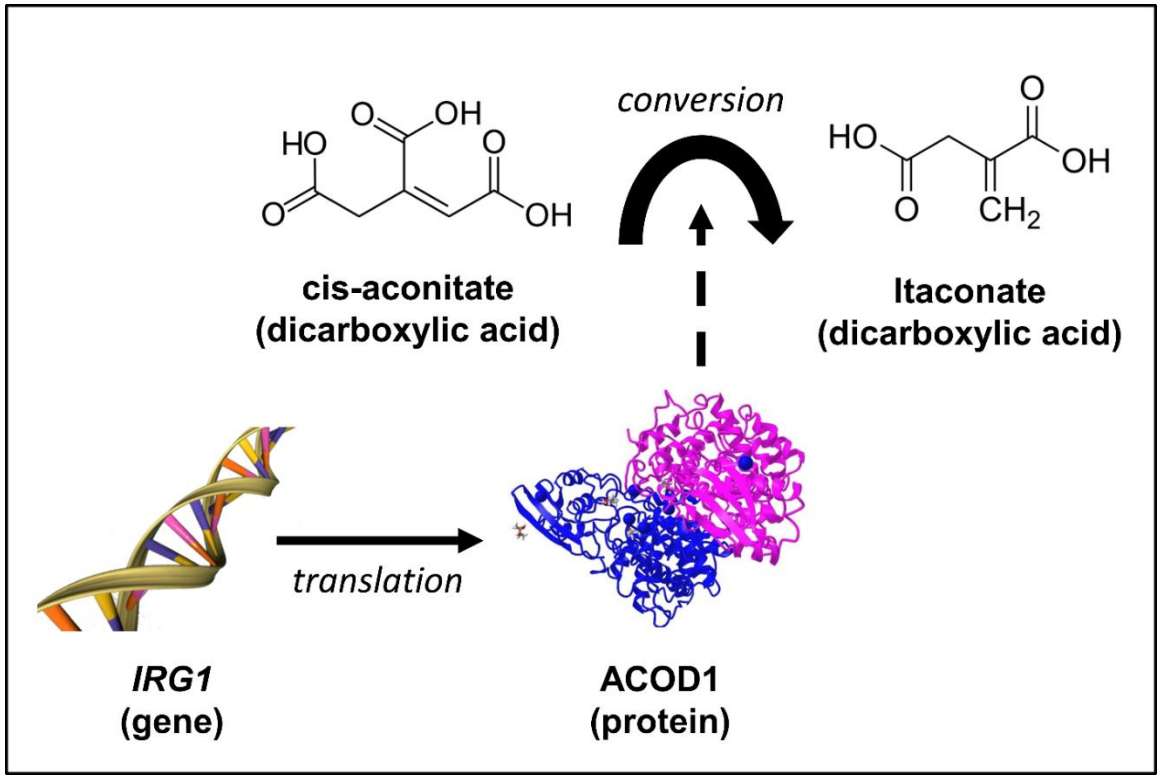

Figure 10. Overview of the evaluation of itaconate on the gene expression, protein and metabolite level.

IRG1 = immune-responsive gene 1; ACOD1 = aconitate decarboxylase 1

Table 12

Colon tissue IRG1 gene expression, ACOD1 protein expression and itaconate tissue levels by patient group

	Type of tissue	**Total group** n=20	**≤50 years of age** n=5	**>50 years of age** n=15
IRG1 gene expression [ΔCT]	**cancer**	N=13 27.4 [95% CI (26.0, 28.7)]	N=4 28.1 [95% CI (24.3; 31.9)]	N=9 27.1 [95% CI (25.4, 28.8)]
IRG1 gene expression [ΔCT]	**normal colon**	N=1 20.4	N=1 20.4	-
ACOD1 protein [ng/ug total protein]	**cancer**	11.8 [95% CI (3.4, 20.2)]	18.4 [95% CI (0, 57.9)]	9.4 [95% CI (4.1, 14.8)]
ACOD1 protein [ng/ug total protein]	**normal colon**	3.7 [95% CI (0.7, 6.7)]	7.4 [95% CI (0, 20.0)]	2.5 [95% CI (0.1, 4.9)]
Itaconate [ng/mg]	**cancer**	216.1 [95% CI (107.8, 324.4)]	214.1 [95% CI (0, 454.9)]	216.9 [95% CI (77.4, 356.3)]
Itaconate [ng/mg]	**normal colon**	153.2 [95% CI (103.4, 203.0)]	231.2 [95% CI (124.4, 338.1)]	125.3 [95% CI 69.6, 181.1)]

IRG1 = Immune-Responsive Gene 1; ACOD1= cis-aconitate decarboxylase 1

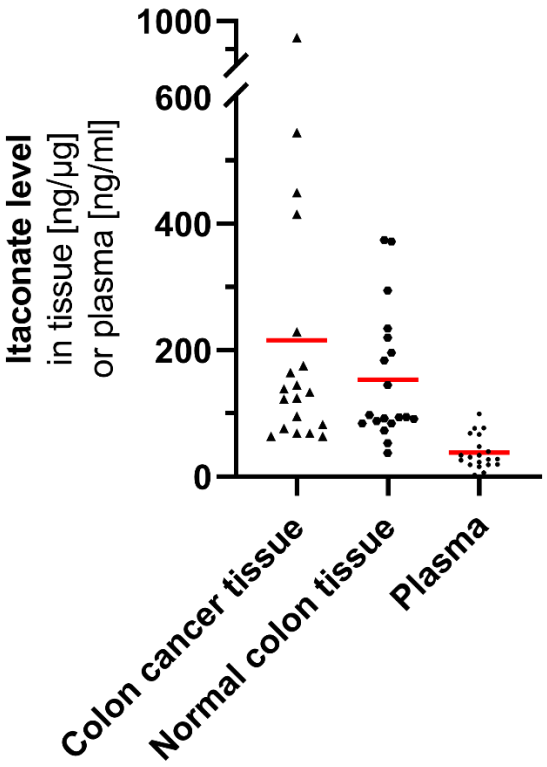

Figure 11. Distribution of itaconate levels with mean values (red) among patient tissue and plasma samples.

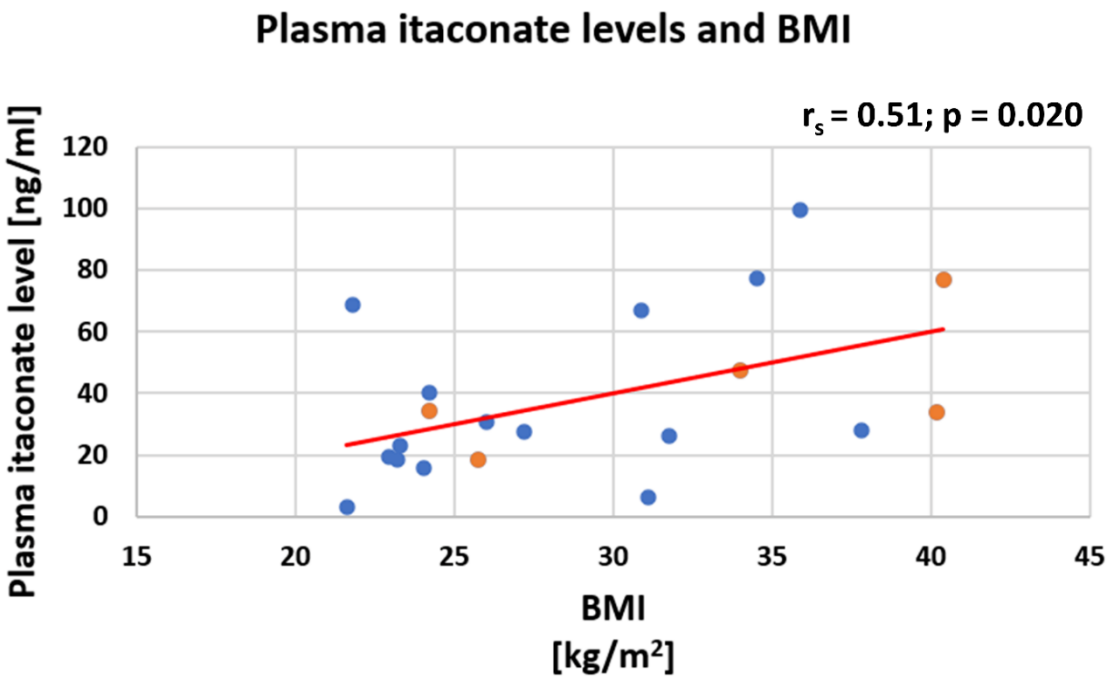

Figure 12. Correlation between plasma itaconate level and Body Mass Index (BMI) among patients.

Levels of patients >50 years of age are shown in blue, patients ≤50 years of age are represented in orange.

BMI = body mass index

iii. Differential gene expression and survival among RNA sequencing data

The TCGA cohort (n=40) involved 48% men and patients with a mean age of 71 (range 40-90). Tumor stage was available for 39 out of 40 patients. Stage I was found in five patients, stage II in 22 patients, stage III in five individuals and stage IV in seven patients. Metadata such as patient age, gender and tumor stage were not available for the EGA dataset (n=69).

Out of 109 patients, IRG1 was expressed in 53% of cancer samples compared to 25% of normal colon tissues ($z = 4.68$, $p=<0.001$). Differential expression analysis showed upregulation of IRG1 in CC tissue relative to paired normal colon samples ($\log_2 FC = 1.41$, corrected $p = 0.03$).

Survival analysis on IRG1 expression (measured as expressed/not expressed) showed to have no effect on survival across 197 CC patients at different stages of tumor progression ($X^2 = 0$, $p = 0.9$). **Table 13** displays the number of events at each stage. No deaths occurred at stage I, and no effect on survival was found for stage II and III (stage II $X^2 = 1.4$, $p = 0.2$; stage III $X^2 = 1.7$, $p = 0.2$). However, for the subset of patients with tumors at stage IV, the risk of death was increased for IRG1 gene expression relative to no expression ($X^2 = 6.3$, $p = 0.01$). Kaplan-Meier curves showing survival rates are shown in **Figure 13**.

Table 13

Number of events at different tumor stages

Stage	Deceased	Living
I	0	31
II	11	67
III	14	51
IV	9	14

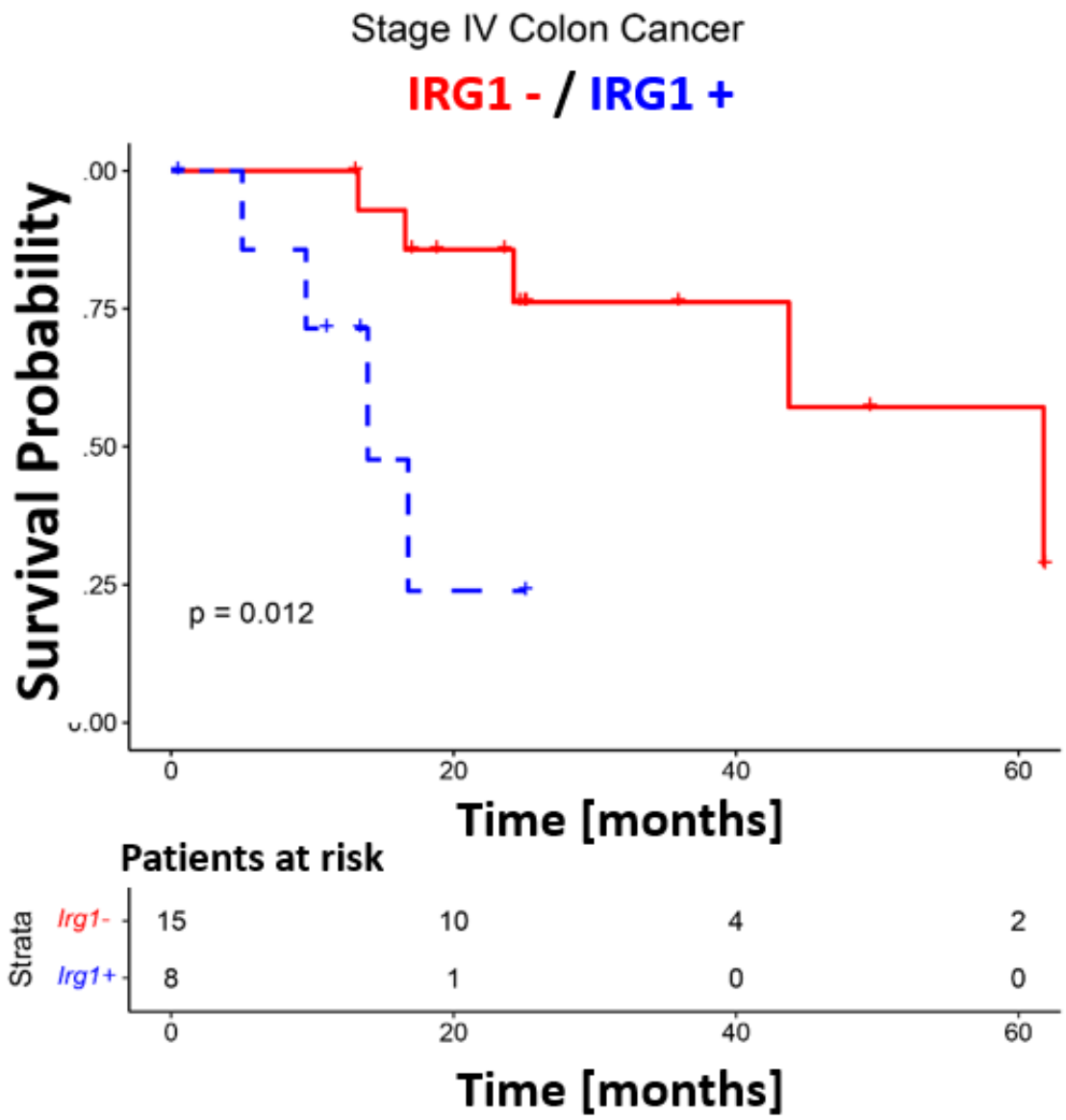

Figure 13. Kaplan-Meier curves for patients with stage IV colon.

IRG1 gene expression (blue, dotted line) versus no IRG1 gene expression (red, solid line).

DISCUSSION

The macrophage-specific metabolite itaconate is a product of the citric acid cycle and serves as an anti-inflammatory mediator decreasing oxidative stress (54, 118, 210). Furthermore, its carcinogenic effects in different types of cancers have previously been described (35, 206, 207). Itaconate therefore has the potential to provide a link between metabolic dysfunction, obesity and cancer.

To the best of our knowledge, data is lacking with respect to IRG1-related expression and itaconate levels in human sporadic CC. The findings of this study demonstrate that itaconate may play a role in patients with sporadic CC and obesity and that it has the potential to play a major role in young patients with the increasing trends in obesity and the increasing incidence of EOCC.

The gene expression of IRG1 encoding the enzyme producing itaconate was detectable in a significantly higher number of cancer samples (65%) compared to paired normal colon tissue (5%). This was confirmed by RNA-seq data showing expression in 53% of cancers versus 25% of paired normal colon samples. Protein expression of ACOD1 confirmed these results demonstrating a significantly higher amount of ACOD1 protein in cancer samples compared to normal colon. These findings indicate that IRG1 is amplified in sporadic colon adenocarcinoma and may serve as an oncogene target in CC. In other types of cancers, such as human glioma, similar expression patterns of ACOD1 protein have been described, correlating with tumor stages and recurrence-free survival (207). As protein expression in these previous reports was classified as either high or low, quantitative levels of ACOD1 protein in glioma tissue were not

reported (207). Monocytes derived from ascites fluid of patients with ovarian carcinoma showed a significantly elevated IRG1 gene expression, enhancing peritoneal tumor progression (35). Furthermore, increased IRG1 mRNA expression has been described in the murine cell lines B16 (melanoma), 3LL (Lewis lung carcinoma) and MC38 (colon adenocarcinoma) (35). These findings suggest that IRG1-related mechanisms mediate tumor-promoting effects and may not be specific for patients with CC. The pivotal role of inflammation and macrophage metabolism in CC, however, points out the particular importance of the immunosuppressive effects of IRG1, and the metabolic mediator itaconate in CC (175, 215). Itaconate directly modifies oxidative phosphorylation and therefore cellular metabolism in TAMs, which is associated with macrophage polarization and induces reactive oxygen production (ROS) (35). ROS can mediate tumor-promoting mechanisms through several transcription factors, such as Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinases (MAPKs) (216). Furthermore, M2-polarized TAMs are associated with precancerous dysplasia in colon polyps and poor patient survival in CC (49, 78). The relationship between itaconate production and an M2-like macrophage phenotype in CC remains to be further investigated.

The metabolic profile of IRG1-related mechanisms and itaconate may provide a link between metabolic dysfunction and obesity and EOCC in young patients. In this study, an association of systemic itaconate and obesity was demonstrated by showing a positive correlation between preoperative plasma itaconate levels and BMI (r_S=0.51; p=0.020). The chronic inflammatory metabolic

state in patients with obesity and CC may be associated with a certain phenotype of peripheral monocytes producing itaconate. A proinflammatory phenotypic switch of adipose tissue-infiltrating macrophages is described, contributing to insulin resistance and diabetes in obesity (217, 218). This mechanism could also be associated with systemic itaconate levels and an increased risk of CC. The demonstrated correlation analysis cannot confirm a causal relationship between these variables, and the size of the studied patient cohort is not sufficient to reveal an empirical correlation. Finding this significant association despite these limitations, however, suggests systemic itaconate levels to be a target of interest in patients with higher BMI and CC.

Further investigating the disparities between patients with sporadic CC >50 years old and young patients with EOCC, a significantly higher level of itaconate was identified in normal colonic tissue of young patients. Cancer tissue itaconate levels did not differ between the two age groups. This finding has not been previously described; however, conclusions are limited due to the small sample size. A significant difference was shown despite the high variability of measured itaconate levels among samples. These findings illustrate that in addition to a general role of itaconate in CC, a specific mechanism in cancer-onset may be related to itaconate in young patients and should be further investigated.

Tumor metabolism and clinical outcome is affected by microsatellite-instability (MSI) and associated with tumor location within the colon in CC (219-221). Furthermore, MSI cancers are more likely to cause an immune response

due to abnormal protein translation (222). This points out another limitation of this study, since patients could not be sorted according to these criteria due to a lack of the relevant data. In addition, measured ACOD1 and itaconate levels could not be correlated with tumor stage due to small sample size.

Differential gene expression analysis for IRG1 revealed a significantly higher expression in cancers compared to normal colon tissue, suggesting a role of *IRG1* in tumor growth and development. A significant impact of IRG1 gene expression in cancer tissue on patient survival was only found in stage IV CC. An effect in earlier cancer stages may be masked due to limited sample size and a generally low expression of *IRG1* that is detected depending on the sensitivity of the respective sequencing technique. Standardized tissue quality and sequencing methods suggest a feasibly comparable pool of data; however, differences in the resolution of gene expression between samples may be impaired.

Itaconate and its related carcinogenic mechanisms could serve as a new target in obese patients with CC and in EOCC. This suggests that TAM metabolism may affect cancer progression through anti-inflammatory mechanisms mediated by itaconate in EOCC.

CHAPTER VII

LEPTIN AND ITACONATE AFFECTING PPARγ IN M2-LIKE MACROPHAGES – A POTENTIAL LINK TO EARLY-ONSET COLON CANCER

INTRODUCTION

The obesity-related hormone leptin circulates systemically as a 146-amino acid glycoprotein that is primarily produced and released by adipocytes (223) (**Figure 14A**). Its role in central appetite and energy regulation is only one facet of this hormone's functions and does not describe its complexity due to the numerous effects of leptin in obesity-related complications, such as type 2 diabetes, cardiovascular disease and hypertension (224). In CRC, leptin has a metabolic impact, affecting cytokine expression in CRC, as well as macrophage polarization (121, 225). Leptin acts as a central mediator of inflammation in CRC inducing proinflammatory cytokine production of TNF-α and/or Interleukin 6 (IL-6) in macrophages and lymphocytes (226). In mouse models, leptin treatment results in reduced CC growth, an increased proportion of proinflammatory M1-like macrophages in CC and increased proinflammatory cytokine production (121, 227). An indirect relationship between obesity and CRC has been reported

due to the association of the function of leptin with several known risk factors of CRC, such as energy intake, sex hormone levels, stress, and inflammatory immune responses (224). A causal link between obesity and CRC through leptin, however, has never been demonstrated (224).

Acting through the JAK2-STAT3 pathway, a key pathway in tumorigenesis and metastasis, leptin increases cell survival and cell growth in CC cells, therefore promoting CC progression (94, 225, 226) (**Figure 15**). The MAPK and the AMPK pathways are also regulated by leptin, both altering gene transcription and inflammatory responses of cells. All three of these pathways affect NFκB activity. An excessive activation of NFκB plays a key role in colorectal carcinogenesis (228).

NFκB is a ubiquitous transcription factor that regulates cytokine, cytokine receptor and adhesion molecule expression in an inflammatory setting, affecting both cancer cells and TAMs (228). The anti-apoptotic effect of leptin on cancer cells is also mediated through NFκB, inducing proliferation, differentiation, metastasis, angiogenesis and chemoradiotherapy resistance in cancer cells (229). PPARγ is an established target in metabolic dysfunction and insulin resistance, also known as the glitazone receptor (230). This protein is a potent inhibitor of NFκB. Low PPARγ expression in CRC is associated with worse clinical outcomes (231, 232). Leptin slightly downregulates PPARγ expression in human macrophages thereby contributing to this expression pattern (233).

As immune responses and inflammation play an increasingly important role in CRC research, macrophages and their metabolic states are coming into

focus of carcinogenic mechanisms and clinical outcomes. The macrophage-specific metabolite itaconate is a dicarboxylic acid derived from cis-aconitate in the Krebs cycle in macrophages (54) (**Figure 14B**). Macrophage activation with lipopolysaccharide (LPS) or other cytokines, such as interferons, can induce itaconate production by the enzyme ACOD1 in the mitochondrial matrix (54). Itaconate is highly polar and is therefore not able to cross cell membranes easily (234). Due to this limitation, membrane-permeable itaconate derivatives, such as OI and DI, are commonly used for in vitro experiments (119, 235). In addition to the effects of leptin, itaconate can enhance NF-κB activity by inducing succinate accumulation and thereby increasing mitochondrial production of ROS (35, 236). The association between leptin, itaconate production and PPARγ expression in macrophages and CRC cells are widely unknown.

The aim is to investigate the effect of the obesity hormone leptin and the membrane-permeable itaconate derivatives OI and DI on M2-like macrophages, their cytokine expression profiles and on PPARγ expression in vitro using qRT-PCR. Dose-response experiments were performed for each treatment by incubating cells with either leptin (n=20), OI (n=20) or DI (n=20) (Sigma-Aldrich, St. Louis, USA). Cellular cytokine expression after treatment with four different doses of each compound (n=5) was determined at four time points including 3, 6, 18 and 24 hours of cell treatment. Detailed methods are described in Chapter IV.

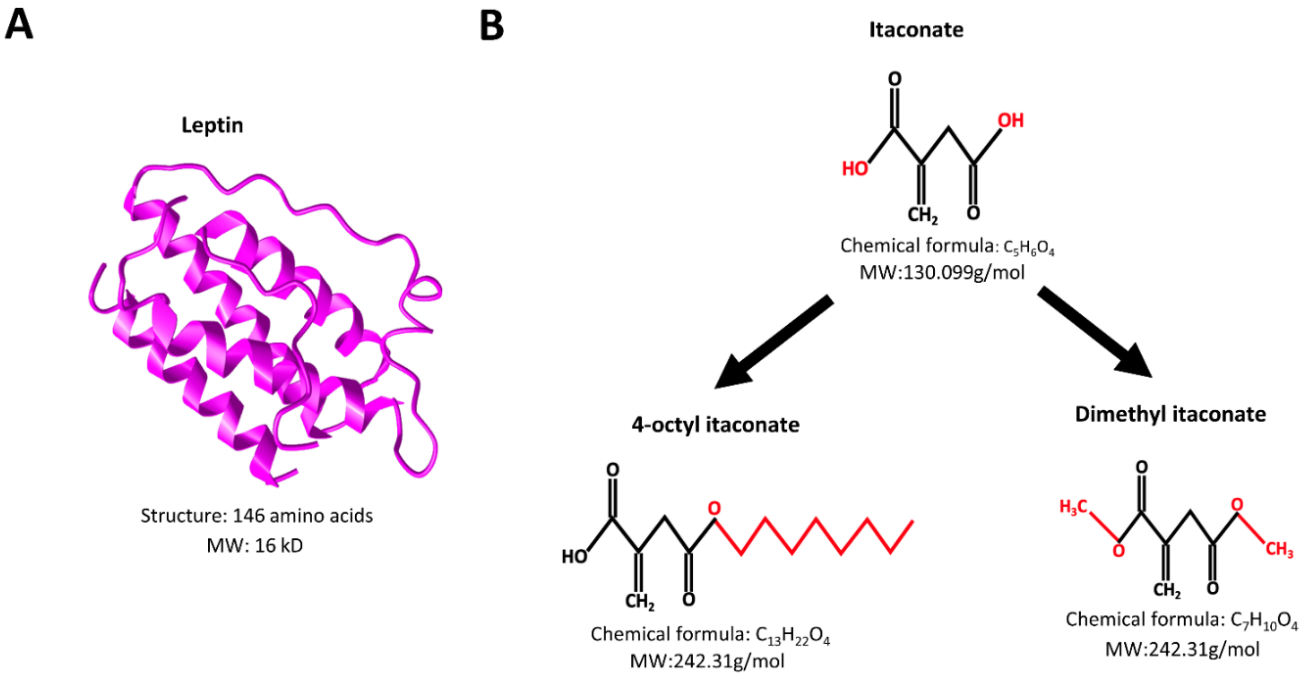

Figure 14. Molecular structures of the macrophage-specific metabolite itaconate, its derivatives and the obesity-related hormone leptin (237).

*(**A**) Quaternary structure of the protein leptin-E 100 that is circulating systemically, affecting organs and tissues (237).*
*(**B**) Fischer projection of itaconate and its derivatives 4-octyl itaconate and dimethyl itaconate with chemical formula and molecular weight. Altered structures between itaconate and its derivatives are shown in red.*

MW = molecular weight; kD = kilodalton

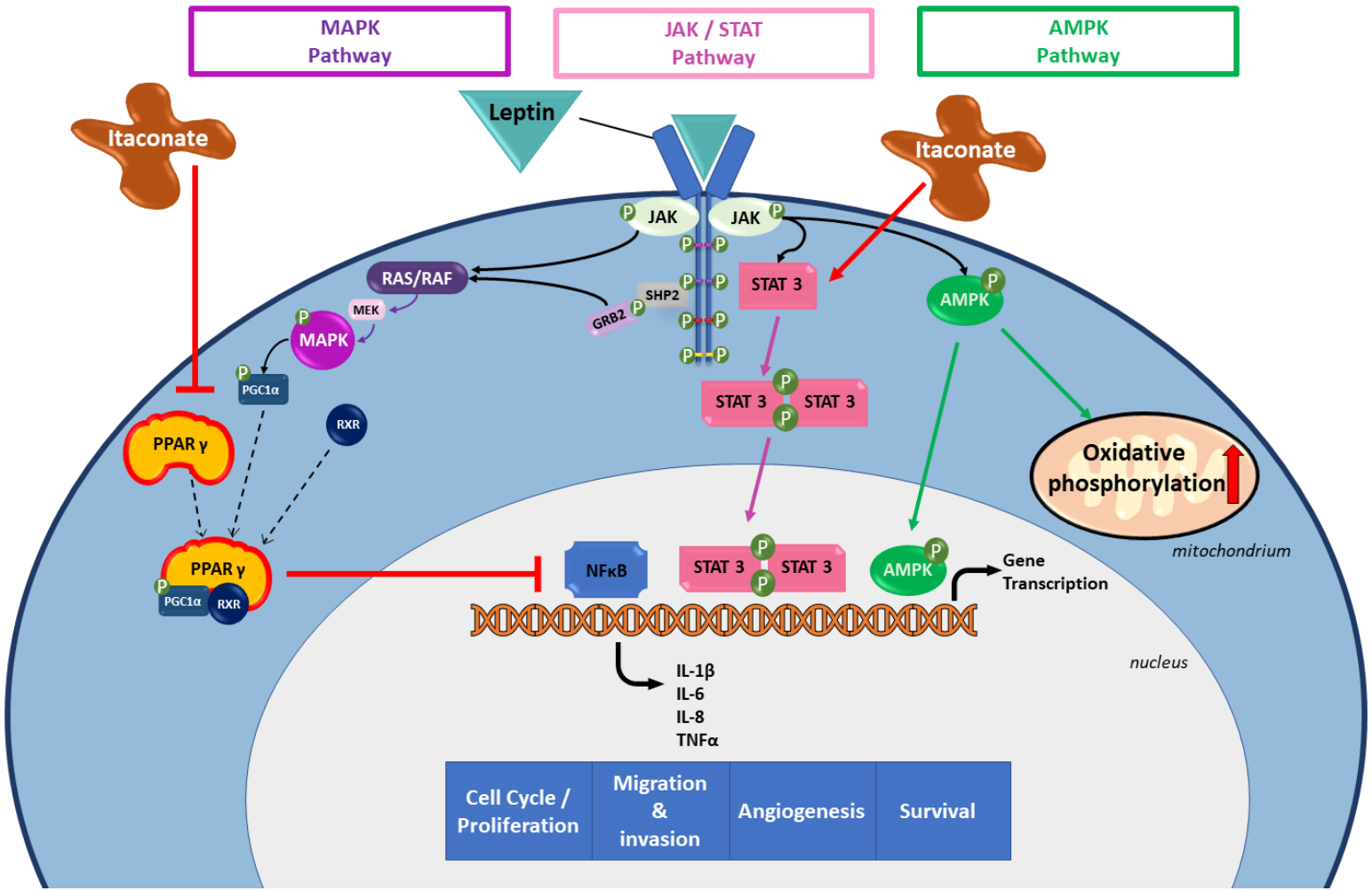

Figure 15. Effects of leptin and itaconate on cellular pathways altering gene transcription, including the MAPK, the JAK/STAT and the AMPK pathway

Legend to Figure 15.

*The three molecular pathways **MAPK (purple)**, **JAK/STAT (pink)** and **AMPK (green)** and their regulation through leptin and its receptor. Molecules in the cytoplasm (light blue) and the nucleus (grey) are shown.*
All three of these pathways alter gene transcription affecting inflammatory responses induced by the NFκB system. MAPK and STAT3 initiate induction of NFκB mediated gene expression, enhancing pro-proliferative and anti-apoptotic mechanisms. On the other hand, AMPK signaling can indirectly inhibit NFκB activity by induction of several downstream factors, such as p53. Furthermore, AMPK plays a key role in macrophage polarization by regulating mitochondrial oxidative phosphorylation and glycolysis.
Itaconate can enhance mechanisms mediated by leptin through activation of the JAK/STAT pathway and downregulation of PPARγ (red arrows).

MAPK = mitogen-activated protein kinase; JAK/STAT = *Janus kinase-signal transducer and activator of transcription; AMPK* = AMP-activated protein kinase; *NFκB = Nuclear factor kappa light chain enhancer of activated B cells; p53 = tumor protein p53; PPARγ = peroxisome proliferator activated receptor gamma; SHP2 = SH2 containing protein tyrosine phosphatase-2; GRB2 = Growth Factor Receptor Bound Protein 2; RAS/RAF* = Rapidly Accelerated Fibrosarcoma-Rat sarcoma protein; MEK = *MAPK/extracellular signal–regulated kinase kinase; PGC1α = peroxisome proliferator-activated receptor gamma coactivator 1-alpha; RXR = retinoid X receptor; IL = interleukin; TNF-α = Tumor Necrosis Factor Alpha*

RESULTS

For detailed methods see Chapter IV.

i. Gene expression in M2-like macrophages

Raw gene expression data, expressed as normalized PCR cycles (ΔCT values), is shown in **Table 14**. Mean ΔCT values for all doses (D1, D2, D3, D4) for treatments with leptin, OI and DI and for each respective gene at all four time points are shown in **Figure 16**.

PPARγ gene expression was not altered in M2-like macrophages treated with either leptin or DI. In contrast, OI treatment resulted in significant downregulation of PPARγ at the highest dose (D4) at 6h (FC -3.78, p=<0.001), 18h (FC -32.67, p=<0.001) and 24h (FC -12.55, p=<0.001) (**Figure 16a**).

Leptin and DI had no significant effect on anti-inflammatory IL-10 expression at all doses and at all time points. OI treatment induced a clear downregulation of IL-10 using the two highest doses (D3 and D4) at all time points (at 3h [FC -54.57 at D3, p=<0.001], at 6h [FC -80.45 at D3, p=<0.001], at 18h [FC -101.13 at D4, p=<0.001] and at 24h [FC -28.84 at D4, p=<0.001]) (**Figure 16b**).

Treatment of cells with leptin consistently upregulated IL-8 expression at all doses with a maximum fold change using the highest dose at 6h (FC 26.35 at D4, p=<0.001) and 18h (FC 12.82 at D4, p=0.006) (**Figure 16c**). DI showed this effect as an early response at 3h (FC 4.44 at D4, p=<0.001) and at 6h (FC 23.26 at D3, p=0.006) (**Figure 16d**). OI had no significant effect on IL-8 expression.

There was no significant effect on CCL18 expression at any dose or time point for leptin, DI or OI treatments.

Proinflammatory IL-1β and IL-6 expression also were not affected in a time- or dose-dependent manner by leptin, DI or OI.

A significant downregulation of proinflammatory TNF-α gene expression was demonstrated following OI treatment at 6h (FC -9.06 at D3, p=<0.001), 18h (FC -103.25 at D4, p=<0.001) and at 24h (FC 36.00 at D3, p=<0.001) (**Figure 16e**).

Table 14

Maximum fold changes (FC) and associated mean ΔCT values in M2-like macrophages for the respective treatment and gene

Gene	Treatment	Time [hours]	Control [mean ΔCT (95% CI)]	D1 [mean ΔCT (95% CI)]	D2 [mean ΔCT (95% CI)]	D3 [mean ΔCT (95% CI)]	D4 [mean ΔCT (95% CI)]	max. FC (dose)	p-value
PPARG	leptin	18	18.25 (17.85 - 18.64)	18.84 (18.48 - 19.20)	18.08 (17.40 - 18.77)	18.11 (17.76 - 18.46)	18.64 (18.28 - 19.00)	-1.51 (D1)	ns
	4-octyl itaconate	**18**	**18.92 (18.50 - 19.34)**	**18.75 (18.40 - 19.11)**	**19.60 (18.75 - 20.46)**	**20.77 (19.80 - 21.74)**	**23.95 (23.72 - 24.17)**	**-32.67 (D4)**	**<0.001**
	dimethyl itaconate	24	18.66 (18.28 - 19.04)	18.60 (18.36 - 18.83)	18.31 (18.02 - 18.60)	18.11 (17.76 - 18.46)	17.89 (17.49 - 18.29)	1.71 (D4)	ns
IL8	**leptin**	**6**	**18.45 (18.31 - 18.58)**	**17.00 (16.68 - 17.32)**	**15.58 (14.31 - 16.84)**	**14.97 (13.86 - 16.07)**	**13.73 (12.62 - 14.84)**	**26.35 (D4)**	**<0.001**
	dimethyl itaconate	**6**	**17.80 (16.66 - 18.95)**	**16.13 (14.62 - 17.64)**	**15.37 (13.73 - 17.01)**	**13.26 (11.01 - 15.50)**	**13.71 (12.44 - 14.97)**	**23.26 (D3)**	**0.006**
CCL18	leptin	18	18.15 (17.86 - 18.44)	18.54 (17.60 - 19.48)	19.22 (18.28 - 20.16)	18.20 (17.74 - 18.65)	18.07 (17.73 - 18.42)	-2.10 (D2)	ns
	4-octyl itaconate	18	16.61 (10.33 - 22.88)	16.19 (10.21 - 22.18)	18.60 (10.88 - 26.33)	16.67 (10.38 - 22.95)	21.14 (14.25 - 28.04)	-21.10 (D4)	ns
	dimethyl itaconate	6	18.85 (18.04 - 19.66)	17.12 (15.97 - 18.26)	17.09 (15.81 - 18.37)	17.19 (16.23 - 18.15)	17.32 (16.48 - 18.17)	3.39 (D2)	ns
IL10	leptin	3	19.05 (18.16 - 19.94)	14.09 (13.71 - 14.47)	16.10 (13.86 - 18.33)	19.71 (19.37 - 20.05)	14.64 (12.28 - 17.01)	31.12 (D1)	ns
	4-octyl itaconate	**18**	**19.06 (16.94 - 21.19)**	**19.58 (17.92 - 21.24)**	**21.72 (19.42 - 24.02)**	**24.12 (22.58 - 25.65)**	**25.72 (23.42 - 28.03)**	**-101.13 (D4)**	**<0.001**
	dimethyl itaconate	18	19.19 (17.18 - 21.19)	18.16 (17.00 - 19.32)	17.76 (16.29 - 19.24)	17.78 (16.25 - 19.30)	18.43 (17.62 - 19.23)	2.69 (D2)	ns
IL1B	leptin	6	21.28 (20.74 - 21.82)	20.61 (19.88 - 21.35)	20.20 (19.34 - 21.05)	19.41 (18.10 - 20.73)	18.04 (16.74 - 19.33)	9.45 (D4)	ns
	4-octyl itaconate	3	18.58 (15.99 - 21.16)	20.41 (20.17 - 20.65)	22.80 (22.46 - 23.14)	22.47 (22.07 - 22.88)	21.18 (20.08 - 22.27)	-18.64 (D2)	ns

	dimethyl itaconate	**24**	**22.01 (21.75 - 22.27)**	**21.70 (20.71 - 22.70)**	**21.08 (20.85 - 21.30)**	**20.48 (19.90 - 21.06)**	**17.84 (17.46 - 18.21)**	**18.00 (D4)**	**<0.001**
IL6	leptin	3	24.23 (20.46 - 28.00)	22.64 (19.11 - 26.17)	21.51 (16.84 - 26.18)	21.39 (18.55 - 24.24)	22.19 (19.26 - 25.12)	7.16 (D3)	ns
	4-octyl itaconate	24	27.56 (26.59 - 28.53)	28.65 (28.02 - 29.29)	28.50 (27.60 - 29.40)	27.01 (24.66 - 29.36)	23.31 (18.97 - 27.66)	19.03 (D4)	ns
	dimethyl itaconate	18	24.99 (20.99 - 29.00)	27.86 (26.86 - 28.87)	28.72 (27.93 - 29.51)	28.27 (27.52 - 29.03)	29.03 (28.25 - 29.80)	-16.45 (D4)	ns
TNFA	leptin	6	18.38 (18.15 - 18.60)	18.15 (17.99 - 18.30)	17.77 (17.53 - 18.01)	17.27 (16.73 - 17.80)	16.72 (16.01 - 17.43)	3.16 (D4)	ns
	4-octyl itaconate	**18**	**18.25 (17.15 - 19.36)**	**19.36 (18.80 - 19.93)**	**20.56 (19.49 - 21.62)**	**22.28 (21.17 - 23.40)**	**24.94 (23.05 - 26.83)**	**-103.25 (D4)**	**<0.001**
	dimethyl itaconate	24	19.17 (18.59 - 19.76)	18.45 (17.48 - 19.43)	18.40 (17.88 - 18.93)	16.76 (16.49 - 17.03)	17.78 (17.56 - 18.00)	5.31 (D3)	ns

Legend to Table 14.

Leptin treatment [ng/ml]:

D1: 100
D2: 300
D3: 500
D4: 1000

4-octyl itaconate [µg/ml]:
D1: 10
D2: 30
D3: 50
D4: 100

Dimethyl itaconate [µg/ml]
D1: 20
D2: 50
D3: 100
D4: 200

LEP = leptin; OI = 4-octyl itaconate; DI = dimethyl itaconate; IL = interleukin; PPARG = peroxisome proliferator activated receptor gamma; TNFA = tumor necrosis factor alpha;

ns = not significant

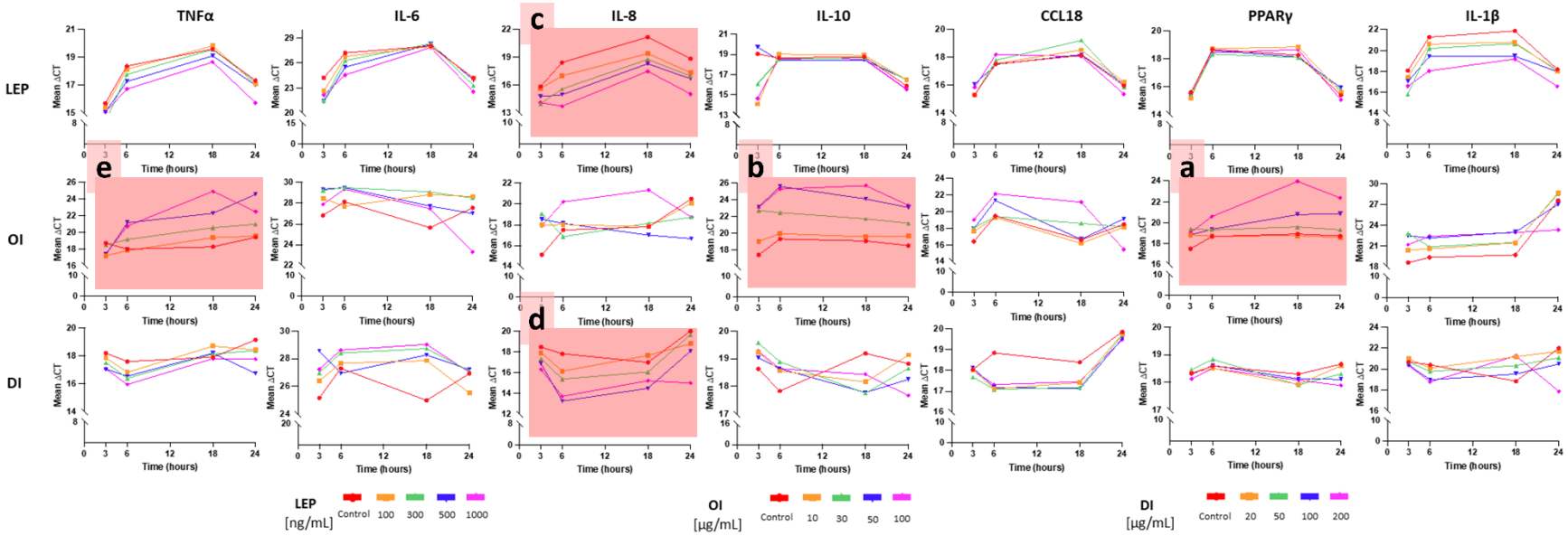

Figure 16. Dose and time response after cell treatment with leptin, 4-octyl itaconate (OI) and dimethyl itaconate (DI)

*Mean PCR gene expression values (ΔCT values) for each cell treatment and for each respective gene, including four different doses (see legend within figure) and four time points (3h, 6h, 18h, 24h). Red highlighting indicates a significant effect (**a,b,c,d,e**).*

LEP = leptin; OI = 4-octyl itaconate; DI = dimethyl itaconate; IL = interleukin; TNFα = tumor necrosis factor alpha; *PPARγ = peroxisome proliferator activated receptor gamma*

ii. Gene expression in M0 macrophages

M0 macrophages were treated with either leptin, OI or DI. Since maximum effects were observed at 18 hours in M2 macrophages, expression levels in M0 macrophages were investigated for this time point specifically. Results are shown in **Table 15**.

Leptin induced an upregulation of IL-8 (p=0.001), TNF-α (p=0.026), IL-1β (p=0.001), CCL18 (p=0.002) and PPARγ (p=0.030) (**Table 15**). Compared to M2-macrophages, only a slight upregulation of IL-8 could be found in M0 macrophages after leptin treatment. Upregulation of TNF-α, IL-1β, CCL18 and PPARγ was significant in M0 macrophages, which could not be demonstrated on M2-like cells (**Table 14, Table 15**).

OI treatment of M0 macrophages led to a downregulation of IL-10 (p=<0.001), TNF-α (p=0.002), IL-1β (p=0.032) and a downregulation of PPARγ (p=0.003) (**Table 15**). Compared to M2-like macrophages, IL-10, TNF-α and PPARγ were downregulated to a lesser extent in M0 macrophages (**Table 14, Table 15**). While no significant changes for IL-1β expression were demonstrated in M2-like macrophages, M0 cells showed a significant downregulation of IL-1β (**Table 14, Table 15**).

DI induced upregulation of IL-8 (p=<0.001), IL-10 (p=0.002), TNF-α (p=<0.001) and IL-1β (<0.001) (**Table 15**). IL-8 upregulation was shown to be higher in M0 macrophages compared to M2-like cells, while IL-1β upregulation was lower in M0 cells (**Table 14, Table 15**). A slight upregulation of IL-10 and TNF-α was

found in M0 with no significant changes among treatment doses in M2-like macrophages (**Table 14, Table 15**).

Table 15

Maximum fold changes (FC) and associated mean ΔCT values in M0 macrophages for the respective treatment and gene

Gene	Treatment	Control [mean ΔCT]	18 hours [mean ΔCT (95% CI)]	mean FC	p-value
IL8	Leptin	19.8	18.2 (17.7 - 18.8)	3.2	0.001
	DI	18.6	13.4 (12.9 - 13.9)	36.8	<0.001
IL10	OI	19.7	24.2 (23.2 - 25.3)	-20.0	<0.001
	DI	19.6	18.9 (18.6 - 19.1)	1.7	0.002
TNFA	Leptin	20.0	19.4 (18.9 - 19.9)	1.5	0.026
	OI	19.7	22.6 (21.5 - 23.6)	-6.5	0.002
	DI	19.4	17.5 (17.3 - 17.8)	3.8	<0.001
IL1B	Leptin	19.5	18.5 (18.2 - 18.8)	1.9	0.001
	OI	19.1	20.7 (19.3 - 22.1)	-2.5	0.032
	DI	18.5	16.3 (15.9 - 16.6)	4.8	<0.001
CCL18	Leptin	24.6	23.7 (23.4 - 24.1)	1.9	0.002
PPARG	Leptin	18.0	17.3 (16.8 - 17.9)	1.6	0.030
	OI	17.3	20.2 (19.0 - 21.3)	-6.0	0.003

LEP = leptin; OI = 4-octyl itaconate; DI = dimethyl itaconate; IL = interleukin; PPARG = peroxisome proliferator activated receptor gamma; TNFA = tumor necrosis factor alpha

DISCUSSION

The expression of the metabolic nuclear transcription factor PPARγ in CRC has a key role in cellular lipid and glucose metabolism and mediates several antineoplastic mechanisms in CRC (231, 238). PPARγ is understood to function as a tumor suppressor, that can serve as a marker of an indolent subset of CRC (232). Itaconate is a metabolic product of the Krebs cycle in TAMs and can alter macrophage cytokine expression as well as their polarization state (54). In metabolic dysfunction and obesity, itaconate can provide a link to EOCRC by enhancing carcinogenic mechanisms that are regulated by obesity-related hormones such as leptin. Identifying this link is the basis for developing new immunotherapeutic targets in CRC. In this study, we provide evidence that itaconate promotes significant downregulation of the tumor suppressor PPARγ in anti-inflammatory M2-like macrophages and upregulation of M2-like macrophage cytokines. In this manner, itaconate promotes carcinogenic mechanisms in EOCRC and obesity-affected pathways through PPARγ.

The itaconate derivatives that have been used in this study demonstrated disparate effects on gene expression in M2-like macrophages. While DI did not seem to alter PPARγ expression, OI resulted in significant downregulation. Furthermore, OI induced a clear downregulation of anti-inflammatory IL-10 expression as well as of proinflammatory TNFα expression. In contrast, DI had a clear impact on IL-8 expression, while OI showed no significant effects. The membrane-permeable derivatives of itaconate that are established in in vitro

investigation, have been reported to not necessarily show similar effects (118, 235). OI and DI are the most commonly used forms of this macrophage-metabolite that are used to provide itaconate intracellularly (118). Mechanisms of intracellular modification of these derivatives, which alter their function and the transmembrane transport mechanisms of itaconate itself are, however, not well understood and are therefore important subjects of current research (119, 235).

Treatment of cells with either leptin, OI or DI led to significant effects on gene expression at different doses over time. Variation of PCR cycle values between plate replicates was shown depending on the respective gene analyzed and upon the compound added for cell treatment. The variability of cellular responses of M2-like polarized macrophages in cell culture demonstrates the high plasticity of this cell type with dynamic and continuous switching between either a more M1-like or more M2-like marker expression. Characterization of the distinct anti-inflammatory M2-like macrophage subtype that was used in this study, however, revealed a clear M2-like polarization state. Despite variability of PCR cycle values between cell plates, a clear pattern of cellular responses could be demonstrated for each respective treatment. The comparison of responses between M2-like TAMs and M0 macrophages demonstrated that the anti-inflammatory phenotype showed a much more pronounced gene expression response to cell treatment than the non-polarized M0 cell type. An exception was found in case of IL-8 expression, which is highly expressed even in untreated M0 macrophages, and which increases following DI treatment of these cells (**Figure 6**). This suggests that leptin and itaconate mediate tumor-promoting cytokine and

marker expression mainly through M2-like TAM responses rather than shifting M0 macrophages into a more anti-inflammatory TAM-like cell type.

A limitation of this study is the focus on TAMs and therefore on only one cell type within the TME. On the other hand, the investigation of individual responses of the macrophage population is important to define their individual contribution to gene expression patterns in CRC. Cell line co-culture models or human CRC organoid cultures are necessary to analyze intercellular mechanisms and paracrine signaling following treatment with leptin and itaconate. Furthermore, this study is limited to the gene expression levels of pathway-related cytokines and PPARγ. After investigating dose- and time-dependent effects in detail, protein expression and further macrophage subtype markers should be determined to identify protein activation and TAM polarization due to leptin and itaconate. In addition, the effects of leptin and itaconate on TAMs should be analyzed using macrophages in different polarization states. Anti-inflammatory M2-like macrophages are the most common subtype of macrophage in more advanced tumor stages and have been shown to be associated with worse clinical outcomes in CRC, such as reduced overall or progression free survival (239). This study limited to TAMs of this M2-like subtype, but the effects on macrophages of other metabolic polarization states, such as M0 or proinflammatory M1-like macrophages, should be evaluated in future experiments.

CHAPTER VIII

LEPTIN, ADIPONECTIN AND ITACONATE AFFECTING GENE EXPRESSION IN COLON CANCER CELLS

INTRODUCTION

Tumor immunology plays an essential role particularly in CRC, since tumor development and progression of this type of cancer are directly linked to inflammatory mediators (240). Cytokine networks can promote cell proliferation, migration and resistance to apoptosis in CRC and are affected by systemic mediators as well as cellular signaling within the TME (94, 240). As part of innate and adaptive immune cells regulating pro- and anti-inflammatory gene expression in tumors, cancer cells are interacting with these cells within the TME and modify their gene expression profile to promote their own growth (240, 241).

The obesity-related hormones leptin and adiponectin can affect cytokine profiles in cancer cells and thereby either inhibit or promote carcinogenic mechanisms (94). A protective function of adiponectin has been postulated for several types of cancers, inhibiting genes that promote apoptosis and cell migration (94, 242). Opposite effects have been found for leptin, affecting the STAT3-NFκB axis, which is critical in CRC (94, 240). The macrophage-specific metabolite itaconate and its cell-permeable derivatives 4-octyl itaconate and

dimethyl-itaconate are strong inflammatory mediators with a potential role in different types of cancers, but their effects on cytokine expression profiles in CRC are poorly understood (54). The acute inflammatory response is critical for the host defense and mediates antitumorigenic effects. In contrast, chronic proinflammatory stress leads to an immunosuppressive environment, exerting predominately anti-inflammatory features (243). Chronic inflammation is a key feature of obesity and a hallmark in CRC that is linked to a high M2/M1 macrophage phenotype ratio and secretion of immunosuppressive cytokines and chemokines by cells within the TME, such as IL-10 and CCL22 (243). This leads to recruitment of T helper cells and regulatory T cells and decreased cytotoxic T cell activity, inhibiting cancer cell apoptosis (243).

CC cells were demonstrated to secrete mediators that modify TAM polarization and cytokine expression, further enhancing tumor growth and progression through IL-6 (18, 243). Leptin, adiponectin and itaconate affect cellular metabolism and marker expression of cancer cells. The two isotypes of adiponectin receptors, adiponectin receptor 1 and 2 (AdipoR1 and AdipoR2), are both expressed in CC cells and are associated with decreased epithelial cell proliferation (244). Leptin receptor expression as well as expression of the actual hormone leptin is increased in CC versus normal colon cells and promotes tumor growth (245, 246). Furthermore, leptin can reduce cytotoxic effects of the common chemotherapeutic agent 5-fluorouracil in CC (247). A tumor inhibiting role of leptin under certain circumstances, however, is still discussed (248).

The aim is to define cytokine and gene expression profiles in CC cells as a response to treatment with the obesity-related hormones leptin and adiponectin and the two derivatives of the macrophage-specific mediator itaconate, OI and DI using qRT-PCR. Detailed methods are described in Chapter IV.

RESULTS

HT-29 CC cells were incubated with either leptin, adiponectin, OI or DI for four different time periods (3, 6, 18, 24 hours). Cells were then harvested, qRT-PCR was performed, and delta CT values were compared to a negative control. Results are shown in **Table 16**.

Leptin induced upregulation of anti-inflammatory IL-8 expression with a maximum fold change (FC) after 6 hours (FC 7.3, p=<0.001). Proinflammatory TNF-α was also upregulated with a maximum FC after 18 hours (FC 5.8, p=0.027) following leptin treatment (**Table 16**).

CC cells treated with adiponectin also showed an upregulation of anti-inflammatory IL-8 with a maximum effect after 3 hours (FC 68.1, p=<0.001). Proinflammatory CXCL10 and TNF-α were upregulated following adiponectin treatment with a maximum fold change after 6 hours, respectively (FC 3.0, p=0.025 and FC 110.7, p=<0.001). The chemokine CCL22 also was significantly upregulated with a maximum after 18 hours (FC 16.3, p=<0.001) (**Table 16**).

OI treatment induced downregulation of anti-inflammatory IL-8 with a maximum FC after 24 hours (FC -4.7, p=<0.001). Proinflammatory CXCL10 was downregulated due to OI treatment and the maximum effect was demonstrated after 24 hours (FC -16.8, p=<0.001). The transcription factor PPARγ showed a maximum downregulation after 24 hours (FC -9.8, p=0.031) (**Table 16**).

DI led to downregulation of proinflammatory CXCL10 with a maximum FC after 24 hours (FC -18.3, p=<0.001). A maximum downregulation of transcription factor PPARγ was shown at 18 hours (FC -1.7, p=0.033) (**Table 16**).

Other genes expressing IL-1β, CCL18, IL-6, IL-10, CD80, IRG1 and NFκB showed no significant changes due to any treatment.

Table 16 (part 1)

Gene expression in HT-29 CC cells (ΔCT) after 3, 6, 18 and hours of treatment

Gene	Treatment	*3 h control* ΔCT [mean ± SEM]	*3 h treated* ΔCT [mean ± SEM]	*3 h* p-value	*6 h control* ΔCT [mean ± SEM]	*6 h treated* ΔCT [mean ± SEM]	*6 h* p-value	Overall effect p-value
CCL22	Leptin	23.9 ± 0.8	24.8 ± 0.9	0.638	26.3 ± 0.8	28.1 ± 0.8	0.178	0.168
CXCL10		24.4 ± 0.4	24.3 ± 0.5	0.817	22.0 ± 0.4	22.6 ± 0.4	0.421	0.492
IL1B		27.3 ± 0.7	26.4 ± 0.7	0.292	24.2 ± 0.7	22.7 ± 0.6	0.292	0.133
IL8		19.6 ± 0.3	18.9 ± 0.3	0.052	**21.3 ± 0.3**	**18.5 ± 0.3**	**<0.001**	**<0.001**
PPARG		16.0 ± 0.3	16.3 ± 0.3	0.854	16.3 ± 0.3	16.2 ± 0.2	0.854	0.720
TNF		29.1 ± 0.7	27.7 ± 0.7	0.218	25.2 ± 0.7	25.2 ± 0.7	0.986	**0.024**
CCL22	Adiponectin	21.6 ± 0.5	21.5 ± 0.5	0.819	**24.3 ± 0.5**	**21.5 ± 0.4**	**<0.001**	**<0.001**
CXCL10		**23.6 ± 0.4**	**22.4 ± 0.4**	**0.044**	**22.8 ± 0.4**	**21.2 ± 0.4**	**0.025**	**0.001**
IL1B		**25.2 ± 0.4**	**21.8 ± 0.4**	**<0.001**	23.2 ± 0.5	21.6 ± 0.5	0.058	**<0.001**
IL8		**20.0 ± 0.4**	**13.9 ± 0.4**	**<0.001**	**18.9 ± 0.4**	**14.6 ± 0.4**	**<0.001**	**<0.001**
PPARG		16.2 ± 0.2	16.0 ± 0.2	0.877	16.1 ± 0.2	16.0 ± 0.2	0.877	0.621
TNF		**25.6 ± 0.5**	**19.9 ± 0.6**	**<0.001**	**27.5 ± 0.5**	**20.7 ± 0.6**	**<0.001**	**<0.001**
CCL22	OI	22.8 ± 1.2	26.9 ± 1.2	0.063	25.8 ± 1.5	25.3 ± 1.3	0.947	0.171
CXCL10		**24.4 ± 0.5**	**26.0 ± 0.5**	**0.045**	25.0 ± 0.6	25.9 ± 0.5	0.241	**<0.001**
IL1B		25.8 ± 0.6	26.0 ± 0.6	0.776	27.6 ± 0.6	28.2 ± 0.6	0.673	0.335
IL8		21.1 ± 0.4	20.8 ± 0.4	0.677	**19.5 ± 0.3**	**20.9 ± 0.4**	**0.004**	**<0.001**
PPARG		**16.8 ± 1.0**	**13.5 ± 1.0**	**0.031**	16.2 ± 0.9	18.5 ± 0.9	0.058	**<0.001**
TNF		**28.2 ± 1.2**	**21.7 ± 1.2**	**0.001**	27.2 ± 1.0	26.0 ± 1.0	0.748	**0.003**
CCL22	DI	24.4 ± 0.5	23.8 ± 0.7	0.732	27.6 ± 0.6	25.4 ± 0.7	0.092	0.176
CXCL10		23.2 ± 0.4	23.4 ±	0.702	22.2 ± 0.4	23.3 ± 0.4	0.076	**<0.001**
IL1B		25.2 ± 0.4	24.8 ±	0.647	25.1 ± 0.4	25.8 ± 0.4	0.357	**<0.001**
IL8		19.3 ± 0.3	19.5 ±	0.796	21.3 ± 0.3	20.9 ± 0.2	0.590	0.312
PPARG		15.8 ± 0.2	16.3 ±	0.233	16.5 ± 0.2	16.5 ± 0.2	0.899	**0.025**
TNF		28.3 ± 0.5	29.6	0.314	27.8 ± 0.5	28.4 ± 0.5	0.371	0.152

Table 16 (part 2)

Gene expression in HT-29 CC cells (ΔCT) after 3, 6, 18 and hours of treatment

Gene	Treatment	*18 h control* ΔCT [mean ± SEM]	*18 h treated* ΔCT [mean ± SEM]	*18 h* p-value	*24 h control* ΔCT [mean ± SEM]	*24 h treated* ΔCT [mean ± SEM]	*24 h* p-value	Overall effect p-value
CCL22	Leptin	26.5 ± 0.7	26.4 ± 0.7	0.893	26.1 ± 0.8	27.9 ± 0.7	0.178	0.168
CXCL10		25.2 ± 0.4	24.5 ± 0.4	0.421	25.0 ± 0.5	24.9 ± 0.5	0.817	0.492
IL1B		26.3 ± 0.6	25.4 ± 0.5	0.292	27.8 ± 0.7	26.5 ± 0.7	0.292	0.133
IL8		**21.2 ± 0.3**	**18.6 ± 0.3**	**<0.001**	**20.2 ± 0.3**	**19.2 ± 0.3**	**0.008**	**<0.001**
PPARG		16.6 ± 0.2	16.9 ± 0.2	0.854	16.1 ± 0.3	16.1 ± 0.3	0.961	0.720
TNF		**28.0 ± 0.7**	**25.5 ± 0.7**	**0.027**	29.4 ± 0.7	28.1 ± 0.7	0.218	**0.024**
CCL22	Adiponectin	**28.7 ± 0.6**	**24.7 ± 0.5**	**<0.001**	21.7 ± 0.5	21.6 ± 0.5	0.819	**<0.001**
CXCL10		21.1 ± 0.6	22.1 ± 0.4	0.155	**23.2 ± 0.5**	**21.9 ± 0.4**	**0.044**	**0.001**
IL1B		23.5 ± 0.6	24.0 ± 0.4	0.488	24.3 ± 0.6	23.1 ± 0.4	0.160	**<0.001**
IL8		18.6 ± 0.6	18.9 ± 0.4	0.653	**19.4 ± 0.5**	**17.3 ± 0.4**	**0.002**	**<0.001**
PPARG		14.9 ± 0.3	14.9 ± 0.2	0.877	15.8 ± 0.3	15.3 ± 0.2	0.655	0.621
TNF		25.3 ± 0.7	26.0 ± 0.5	0.440	26.4 ± 0.6	27.0 ± 0.5	0.440	**<0.001**
CCL22	OI	25.5 ± 1.3	25.6 ± 1.3	0.947	26.9 ± 1.2	25.6 ± 1.3	0.947	0.171
CXCL10		**26.4 ± 0.6**	**28.2 ± 0.5**	**0.045**	**25.4 ± 0.5**	**29.4 ± 0.5**	**<0.001**	**<0.001**
IL1B		25.8 ± 0.7	27.3 ± 0.6	0.360	25.6 ± 0.7	26.9 ± 0.6	0.360	0.335
IL8		**19.4 ± 0.4**	**21.6 ± 0.3**	**<0.001**	**20.9 ± 0.4**	**23.1 ± 0.4**	**<0.001**	**<0.001**
PPARG		**16.6 ± 0.9**	**19.3 ± 0.9**	**0.040**	**16.1 ± 0.9**	**19.4 ± 0.9**	**0.031**	**<0.001**
TNF		26.8 ± 1.0	27.8 ± 1.2	0.748	28.0 ± 1.2	27.7 ± 1.2	0.890	**0.003**
CCL22	DI	29.5 ± 0.5	28.9 ±	0.732	26.8 ± 0.5	27.0 ± 0.6	0.771	0.176
CXCL10		**24.1 ± 0.4**	**25.9 ±**	**0.002**	**24.6 ± 0.4**	**28.8 ± 0.4**	**<0.001**	**<0.001**
IL1B		25.5 ± 0.4	25.2 ±	0.647	**25.9 ± 0.4**	**28.3 ± 0.3**	**<0.001**	**<0.001**
IL8		21.1 ± 0.3	21.0 ±	0.796	19.9 ± 0.3	19.1 ± 0.3	0.264	0.312
PPARG		**16.4 ± 0.2**	**17.1 ±**	**0.033**	15.9 ± 0.2	16.3 ± 0.2	0.234	**0.025**
TNF		28.3 ± 0.5	28.9	0.371	27.6 ± 0.6	28.7 ± 0.5	0.314	0.152

Legend to Table 16.

Significant differences in expression and significant overall p-values are marked bold.

SEM = standard error of the mean

DISCUSSION

Obesity-related hormones and itaconate significantly change the gene expression patterns in CC cells and can thereby promote tumor growth, progression and recurrence.

The hormones leptin and adiponectin are reported to have contrary roles in CRC progression, with leptin inducing cancer progression and adiponectin inhibiting tumor growth in vitro (94). Investigating cytokine expression profiles that can affect CC development, however, both cytokines induce pro- and anti-inflammatory marker expression to a certain extent. Anti-inflammatory IL-8 production is a key factor in CRC and associated with enhanced tumor growth, progression and recurrence through autocrine effects on cancer cells (249). Furthermore, IL-8 promotes angiogenesis in CC and mediates proinflammatory effects by functioning as a chemoattractant for neutrophils inducing the JAK3 pathway (249, 250). Leptin significantly upregulated IL-8 expression in CC cells, but adiponectin showed a much higher cellular response with a 68-fold upregulation following treatment. A similar cellular response was found for TNF-α, with adiponectin inducing expression to a much higher extent compared to leptin (111-fold versus 6-fold). This suggests that adiponectin might have tumor promoting abilities by upregulating IL-8 expression and inducing proinflammatory responses through TNF-α in advanced cancer stages.

Both itaconate derivatives downregulated proinflammatory CXCL10 expression, which is a key factor of the anti-tumor T-cell response in CRC (251). Common

chemotherapies in CRC upregulate CXCL10 expression through IFN-γ signaling by DNA damage, which induces cytotoxic T cell recruitment (251, 252). Itaconate can reduce proinflammatory mechanisms and T cell infiltration and therefore anti-tumorigenic activity by downregulating CXCL10.

A prognostic factor in CRC that is associated with patient survival is expression of the tumor suppressor PPARγ. Both derivatives OI and DI can exert tumor enhancing effects with poor patient survival by downregulating PPARγ, with OI showing a higher impact (232).

CCL22 directly induces tumor cell migration and is therefore associated with tumor promoting effects in CC (253). Adiponectin was the only treatment that significantly affected CCL22 expression by upregulating it 16-fold in CC cells. This suggests that adiponectin can enhance tumor progression and metastasis in later stages of the disease.

A major limitation of this work is that effects were only shown in vitro using one CC cell line derived from Duke's C stage human colon adenocarcinoma. Furthermore, cellular responses within the TME are affected by other types of cells that alter the overall effect on tumor development (254). Investigating the response of only one cell type, however, is the basis for coculture models and functional studies using organoid cancers mimicking the TME.

CHAPTER IX

LEPTIN AND ADIPONECTIN AFFECTING THE NOTCH4-GATA4-IRG1 AXIS – A COCULTURE MODEL AND GENOMIC ANAYLSIS

INTRODUCTION

The NOTCH4-GATA4-IRG1 axis shares a network of genes involved in CRC, with the potential to define macrophage metabolism and obesity-related EOCRC. GATA4 is associated with both anti-inflammatory IRG1 and NOTCH4 expression, which in turn are affected by the obesity-related hormone leptin (**Figure 3**). The role of the NOTCH4-GATA4-IRG1 axis in EOCRC as an immunotherapeutic target has yet to be investigated.

Expression of the anti-inflammatory metabolite itaconate by the enzyme encoded by IRG1 in macrophages was shown to be peroxisome proliferator-activated receptor-γ (PPARγ)-dependent (212) (**Figure 3**). Downregulation of PPARG led to increased IRG1 expression in peritoneal mouse macrophages, demonstrating that PPARγ is a regulator of macrophage metabolism (212). Additionally, PPARγ plays a pivotal role in epithelial cell differentiation, and decreased levels of PPARG expression in CRC have been demonstrated to enhance CRC progression (255, 256) (**Table 4**). The ability to affect macrophage metabolism

and CRC progression makes PPARγ a potential key regulator in TAM-mediated CRC development and EOCRC.GATA4 is another gene affecting IRG1 expression, while its definitive role in CRC has yet to be clarified. In pretreated mouse embryonic fibroblasts, GATA4 induced a decrease in IRG1 expression (257) (**Figure 3**). While there is currently no known direct interaction for GATA4 and PPARG, an interaction of GATA family proteins with (PPAR)-binding protein (PBP) in murine embryonic development has been demonstrated (258). Furthermore, GATA4 expression is associated with altered expression of ATP-binding cassette (ABC) transporters, such as the subfamily G member 5 and 8 (ABCG5/ABCG8) (259) (**Table 4, Figure 3**). In GATA4-deficient murine enterocytes, ABCG5 expression was decreased, suggesting a positive association between these two genes (259). ABCG5 expression is common in tumor buds of patients with node-negative CRC and is associated with poor prognosis (260). This might also have a potential role in node-positive cancers requiring chemotherapy, as ABC transporters can regulate chemotherapy resistance by transporting chemotherapeutic agents out of cancer cells (261). This mechanism is assumed to promote 5-fluorouracil resistance in CRC with ABCG5 overexpression (261). Defects in the leptin axis in a murine model affected ABC transporter expression in the liver, leading to decreased excretion of biliary cholesterol (262). The effects of leptin on ABCG5/ABCG8 expression in CRC, however, are poorly understood.

An interplay of GATA4 and Notch signaling was demonstrated in murine Leydig cells, showing downregulation of GATA4-dependent gene expression involved in

steroidogenesis through Notch signaling pathways (263) (**Figure 3**). The impact of GATA4 on NOTCH4 expression in enterocytes has yet to be investigated. Expression of Notch-induced transcription factors, such as HEY1, are increased by Delta-like 4 (DLL4) in human lymphatic endothelial cells (264) (**Figure 3**). DDL4 is a membrane-bound ligand in Notch signaling, targeting NOTCH1 and NOTCH4 (265). DLL4 is of interest in CRC research, since its expression is enhanced by vascular endothelial growth factor α (VEGFA) and hypoxia, two factors enhancing tumor progression (265).

In human coronary artery endothelial cells, VEGFA and NOTCH4 expression showed a positive association (266). In breast cancer, high expression levels of leptin, IL-1, VEGF and Notch proteins were associated with metastasis and poor survival (267). Leptin has the potential to enhance these mechanisms by inducing DLL4 as well as NOTCH4 expression in murine breast cancer cells (267) (**Figure 3**). Another tumor promoting gene associated with NOTCH4, Serine protease inhibitor family E member 1 (SERPINE1), is currently discussed to function as a novel therapeutic target in CRC (268). In murine epithelial breast cancer cells, however, NOTCH4 reduced SERPINE1 expression (269) (**Figure 3**). SERPINE1 is the gene encoding for plasminogen activator inhibitor-1 (PAI-1), which is involved in cell proliferation and decreased apoptosis. It is upregulated in CRC tissue and was shown to be associated with relapse in CRC patients (270) (**Table 4**).

Tumor growth and metastasis are also promoted by enzymes responsible for tissue remodeling and cell migration such as MMPs (271) (**Table 4, Figure 3**).

Notch signaling was positively associated with MMP2 expression in cardiac tissue through HEY1 in an embryonic mouse model investigating epithelial to mesenchymal transition (EMT) (272). In CRC, EMT-associated proteins were also shown to be regulated by MMP2, with increasing expression during tumor progression (271). Furthermore, in tissue remodeling mediated by rat cardiac fibroblasts, it was demonstrated that leptin levels can upregulate MMP2 expression and cell migration (273) (**Figure 3**). In human breast cancer cells, leptin induced SERPINE1 and MMP2 expression (274). The effects of obesity-related hormones on EMT and CRC progression in obese patients, however, deserve further study.

Both SERPINE1 and MMP2 expression are tightly associated with upregulation of proinflammatory cytokine expression. As CRC is a cancer type that is tightly associated with chronic inflammation, inflammatory cytokines that are produced by either cancer cells or other cells of the tumor microenvironment play a crucial role in tumor progression (215). IL-1β, IL-6 and TNF-α are central cytokines in CRC that are associated with the NOTCH4-GATA4-IRG1 axis (**Figure 3**)**.** IL-1β is a proinflammatory cytokine that is elevated in cancer tissue of patients with metastatic CRC and it can induce cancer cell proliferation, promote myeloid cell invasion, and thereby enhance tumor development (102). IL-1β expression is induced by activation of NFκB and is predominantly produced by myeloid cells in the TME (102, 275). This brought IL-1β and associated pathways into focus for new immunotherapeutic treatment strategies (275). Adiponectin can increase IL-1β expression in macrophages, while leptin was demonstrated to induce IL-1β in

murine colon (276) (**Figure 3**). IL-6 and TNF-α are other proinflammatory cytokines induced by leptin, that in turn have been shown to upregulate leptin production (276, 277). Adiponectin mediates anti-carcinogenic effects by suppressing CC cell proliferation due to IL-6 or TNF-α (278). Both these cytokines in turn reduce adiponectin production in breast cancer, while PPARγ agonists increase adiponectin levels (279).

Anti-inflammatory IRG1 expression is negatively correlated with proinflammatory cytokines, such as IL-1β, IL-6 and TNF-α (280).

The aim of this work is to determine the effects of obesity-related leptin and adiponectin on the NOTCH4-GATA4-IRG1 axis using a coculture model of M2-like macrophages and HT-29 CC cells. Furthermore, a genomic analysis of this pathway is performed using human RNA-seq data, and specific gene-related patient survival is investigated. Detailed methods are described in Chapter IV.

i. Inflammatory marker expression and the NOTCH4-GATA4-IRG1 axis in an M2-like macrophage / HT-29 colon cancer coculture model

THP-1 monocytes were differentiated and polarized into M2-like macrophages within 14 days as previously described (182). After combining M2-like macrophages with HT-29 CC cells, cocultures were treated with either 300 ng/ml of leptin or 20 μg/ml of adiponectin for 6 hours and 18 hours, harvested and qRT-PCR was performed. Results are shown in **Table 17**.

1. Leptin in M2-like macrophages and HT-29 CC cells

Leptin treatment induced a significant upregulation of the M1-like cell surface marker CD80 in M2 macrophages in coculture with a maximum at 6 hours (FC 11.3, p=<0.001) (**Table 17**). The M2-like marker CD206 was downregulated at 18 hours of leptin treatment (FC -3.8, p=<0.001).

PPARγ was slightly downregulated in M2-like macrophages and CC cells at 18 hours, respectively (FC -1.3, p=0.011 and FC -0.9, p=0.027). A slight downregulation of NFκB was demonstrated in CC cells, with a maximum at 6 hours (FC -1.5, p=0.004). In M2-like macrophages, however, NFκB expression was considerably upregulated with a maximum FC of 7.7 at 6 hours (p=<0.001) (**Table 17**).

Investigating cytokine expression, IL-6 was upregulated 39-fold in M2 macrophages at 6 hours of leptin treatment (FC 39.4, p=<0.001). IL-6 was not expressed in CC cells at any time. Proinflammatory IL-1β expression in M2 macrophages was maximally upregulated at 6 hours (FC 20.8, p=<0.001), with no difference of expression due to leptin in CC cells. Proinflammatory CCL22 showed a slight upregulation in macrophages at 6 hours only and no changes in CC cells (FC 2.0, p=0.002). CCL18 was significantly downregulated at 18 hours of leptin treatment (FC -2.1, p=0.005).

Anti-inflammatory IL-8 was upregulated in both M2 macrophages and HT-29 cells, with macrophages showing a much higher response compared to CC cells (FC 51.5, p=<0.001 versus FC 1.2, p=0.023) (**Table 17**). IL-10 expression was only demonstrated in M2-like macrophages and showed a slight downregulation at 18 hours (FC -1.8, p=0.001).

SERPINE1 was significantly downregulated in both cell types at 18 hours of leptin treatment, with macrophages showing a -2.3-fold and cancer cells a -1.7-fold downregulation (p=0.023 and p=0.011) (**Table 17**). Macrophage-specific IRG1 expression was maximally upregulated at 6 hours (FC 14.6, p=<0.001). GATA5 and ABCG8 were not expressed in M2-like macrophages. Expression of GATA4 and TNF-α did not change due to leptin treatment (**Table 17**).

GATA4, ABCG8, IL-6, IL-10, CCL-18 and the macrophage markers CD80, CD206 and IRG1 were not expressed in HT29 CC cells. TNF-α, IL-1β and CCL22 showed no significant changes due to leptin treatment (**Table 17**).

Table 17

Maximum significant changes of gene expression in M2-like macrophages and HT-29 CC cells following leptin treatment (300 ng/ml)

	Cell type in coculture	Gene	Control [mean ΔCT]	max. effect [mean ΔCT (95% CI)]	max. effect [FC]	time point of max. effect [hours]	p-value
Leptin	M2	CD80	20.3	16.8 (16.7 - 16.9)	11.3	6	<0.001
	M2	CD206	18.8	20.6 (20.1 - 21.2)	-3.8	18	<0.001
	M2	PPARG	18.4	18.8 (18.6 - 19.0)	-1.3	18	0.011
	M2	NFKB	17.7	14.8 (14.5 - 15.0)	7.7	6	<0.001
	M2	IL6	29.1	23.8 (22.9 - 24.8)	39.4	6	<0.001
	M2	IL1B	18.8	14.4 (14.2 - 14.7)	20.8	6	<0.001
	M2	CCL22	19.4	18.4 (18.1 - 18.7)	2.0	6	0.002
	M2	CCL18	15.6	16.6 (16.1-17.1)	-2.1	18	0.005
	M2	IL8	17.9	12.3 (11.8 - 12.8)	51.5	6	<0.001
	M2	IL10	17.9	18.8 (18.6 - 19.0)	-1.8	18	0.001
	M2	SERPINE1	27.2	28.3 (27.5 - 29.1)	-2.3	18	0.023
	M2	IRG1	25.5	21.7 (21.1 - 22.2)	14.6	6	<0.001
	HT-29	GATA5	25.5	27.5 (25.7 - 29.3)	-5.7	6	0.032
	HT-29	PPARG	16.6	17.0 (16.9 - 17.1)	-1.3	18	0.002
	HT-29	NFKB	16.6	17.3 (17.0 - 17.5)	-1.5	6	0.004
	HT-29	IL8	19.9	19.6 (19.4 - 19.9)	1.2	6	0.023
	HT-29	SERPINE1	21.4	22.2 (21.7 - 22.7)	-1.7	18	0.011

Upregulations are highlighted in green, downregulations in red.

FC = fold change

2. Adiponectin in M2-like macrophages and HT-29 colon cancer cells

Treatment of cells with adiponectin in coculture showed a significant upregulation of the M1-marker CD80 in macrophages, while the anti-inflammatory M2-like marker CD206 was slightly upregulated as well (FC 7.2, p=<0.001 and FC 1.9, p=0.002) (**Table 18**).

PPARG was downregulated in macrophages following adiponectin treatment, with no changes in expression in HT-29 CC cells (FC -2.4, p=<0.001) (**Table 18**).

In M2-like macrophages, adiponectin upregulated NFκB expression (FC 5.8, p=<0.001) as well as all targeted pro- and anti-inflammatory cytokines, such as IL6 (FC 14.9, p=<0.001), IL-1β (FC 9.6, p=<0.001), CCL22 (FC 2.6, p=0.002), CCL18 (FC 2.0, p=<0.001) and IL-10 at 6 hours (FC 2.2, p=0.001) and IL-8 at 18 hours (FC 21.3, p=<0.001). NFκB was maximally upregulated in HT-29 CC cells at 6 hours (FC 1.5, p=<0.001), while IL-1β and CCL22 were maximally downregulated in HT-29 following adiponectin at 6 hours (FC -4, p=0.033 and FC -2.8, p=0.023). SERPINE1 expression was downregulated in macrophages and upregulated in HT-29 cells (FC -4.4, p=0.010 at 6 hours and FC 1.2, p=0.019 at 18 hours).

Macrophage-specific IRG1 was maximally upregulated (FC 5.3, p=0.032) at 18 hours. GATA5 expression was demonstrated to be downregulated in CC cells after 6 hours (FC -2.6, p=0.029).

Table 18

Maximum significant changes of gene expression in M2-like macrophages and HT-29 CC cells following adiponectin treatment (20µg/ml)

	Cell type in coculture	Gene	Control [mean ΔCT]	max. effect [mean ΔCT (95% CI)]	max. effect [FC]	time point of max. effect [hours]	p-value
Adiponectin	M2	CD80	21.2	18.5 (17.8 - 19.1)	7.2	6	<0.001
	M2	CD206	20.6	19.7 (19.3 - 20.0)	1.9	6	0.002
	M2	PPARG	17.9	19.1 (19.0 - 19.3)	-2.4	6	<0.001
	M2	NFKB	17.6	15.1 (14.6 - 15.7)	5.8	6	<0.001
	M2	IL6	28.8	25.0 (24.3 - 25.7)	14.9	6	<0.001
	M2	IL1B	19.6	16.4 (15.9 - 16.8)	9.6	6	<0.001
	M2	CCL22	20.3	19.0 (18.5 - 19.4)	2.6	6	0.002
	M2	CCL18	17.4	16.4 (16.2 - 16.6)	2.0	6	<0.001
	M2	IL8	19.5	15.1 (14.7 - 15.5)	21.3	18	<0.001
	M2	IL10	18.6	17.5 (17.1 - 17.8)	2.2	6	0.001
	M2	SERPINE1	23.3	25.2 (24.0 - 26.3)	-4.4	6	0.010
	M2	IRG1	28.2	26.0 (24.2 - 27.8)	5.3	18	0.032
	HT-29	GATA5	24.8	26.1 (25.0 - 27.1)	-2.6	6	0.029
	HT-29	NFKB	17.2	16.6 (16.4 - 16.8)	1.5	6	<0.001
	HT-29	IL1B	25.1	26.6 (25.3 - 28.0)	-4	6	0.033
	HT-29	CCL22	25.9	27.3 (26.4 - 28.3)	-2.8	6	0.023
	HT-29	SERPINE1	22.5	22.2 (21.9 - 22.4)	1.2	18	0.019

Upregulations are highlighted in green, downregulations in red.

FC = fold change

ii. Patient samples and genomic analysis

Leptin and adiponectin protein levels were measured in plasma, CC tissue and paired normal colon tissue from 20 sporadic CC patients with class I-III obesity.

1. Leptin and adiponectin in patients with sporadic colon cancer

There was a high variability of leptin tissue levels and no significant difference in mean leptin levels between normal and cancer tissue (51.9 ± 52.7 pg/mg versus 41.1 ± 54.7 pg/mg; p=0.765). The mean adiponectin level in normal colon tissue was, however, higher than in CC tissue (32.6 ± 13.3 ng/ug versus 24.2 ± 9.4 ng/ug; p=0.005). The subgroup of patients >50 years of age showed a significant difference in adiponectin tissue concentration between normal colon and CC (37.6 ± 10.4 ng/ug versus 26.7 ± 9.7 ng/ug; p=0.003), while no difference could be demonstrated in young individuals ≤50 years (17.9 ± 5.1 ng/ug versus 19.5 ± 11.4 ng/ug; p=0.625).

There was a positive correlation between plasma leptin levels and BMI (r_s=0.6; p=0.003) was observed, and a negative correlation between plasma adiponectin levels and BMI (r_s=0.7; p=<0.001).

2. Genomic analysis on gene expression in patients with colon cancer

RNA-seq data on 157 sporadic CC patients with matched normal colon tissue was analyzed to include 18,884 genes. Differential gene expression, tissue interaction and patient survival analysis for target genes were performed. IRG1 showed the lowest mean overall expression and therefore no significant changes (**Table 19**). Differential gene expression demonstrated a 2-fold upregulation of the transcription factor GATA4 in CC versus normal colon tissue (p=0.010) (**Table 19**). A significant age by tissue interaction was found for GATA4 with a 3-fold increase in GATA4 expression in CC tissue of young patients with EOCC compared to CC older patients (p=0.045). GATA5 was differentially expressed showing a 4-fold downregulation in CC versus normal colon tissue (p=<0.001), SERPINE1 was 1-fold downregulated (p=0.015), and IL-1β was 2-fold upregulated (p=0.020) (**Table 19**).

Six target genes showed significant age by gene interaction (**Table 19**). All of these genes were significantly upregulated in EOCC. GATA4 and SERPINE1 demonstrated the highest increase in expression among young CC patient samples (3-fold and 2-fold, p=0.045 and p=0.001, respectively) (**Table 19**). Analyzing BMI by tissue interaction, a significant upregulation of GATA5 was demonstrated in obese versus normal-weight (p=0.030) patients. Another gene showing a significant BMI by tissue interaction was ABCG5, showing higher expression in obese patients (p=0.003) (**Table 19**).

Survival analysis of the genes with significant age or obesity by tissue interactions revealed that high GATA4, GATA5 and HEY1 expression were associated with decreased overall patient survival, (p=0.039, p=0.015 and p=0.036, respectively) (**Figure 17**). ABCG5, DLL4, NOTCH4, SERPINE1 and VEGFA showed no significant impact on patient survival. SERPINE1 and VEGFA showed trends towards decreased patient survival in the case of high gene expression (**Figure 17**).

Table 19

Gene expression (normalized read counts), differential gene expression and age and BMI by tissue interaction of genes within the NOTCH4-GATA4-IRG1 axis

Gene	Mean overall reads	Differential expression (Cancer vs. Normal) [log2 FC]	p-value	Age by tissue interaction	p-value	BMI (>40 kg/m^2) by tissue interaction	p-value
ABCG5	39.7	**-1.3**	**0.005**	0.2	0.927	**2.1**	**0.003**
ADIPOQ	404.6	**-2.1**	**0.025**	-1.5	0.496	0.5	0.894
DLL4	1014.7	**0.4**	**0.018**	**0.8**	**0.011**	-0.4	0.420
GATA4	53.6	**1.9**	**0.010**	**2.6**	**0.045**	2.2	0.149
GATA5	25.6	**-3.7**	**<0.001**	0.5	0.751	**2.1**	**0.030**
HEY1	355.1	**-1.3**	**<0.001**	**0.8**	**0.011**	-0.1	0.877
IL1B	561.7	**2.2**	**<0.001**	-0.7	0.501	-0.3	0.893
IL6	290.8	**-1.2**	**0.014**	0.5	0.718	-0.7	0.619
IRG1	1.4	0.1	0.945	-0.9	0.706	-0.1	0.978
LEP	141.4	**-2.2**	**0.005**	0.6	0.803	0.9	0.718
MMP2	14653.7	-0.4	0.147	0.2	0.852	-0.4	0.670
NOTCH4	1117.5	**-1.0**	**<0.001**	**1.0**	**0.007**	-0.6	0.295
PPARA	2320.5	0.1	0.643	-0.3	0.275	0.0	0.981
PPARG	2175.9	**0.8**	**0.005**	-0.1	0.961	0.1	0.921
SERPINE1	2996.2	**-1.0**	**0.015**	**2.1**	**0.001**	-0.4	0.789
VEGFA	6319.9	**0.8**	**<0.001**	**0.7**	**0.017**	-0.4	0.450

Significant results are highlighted.

FC = fold change; BMI = body mass index

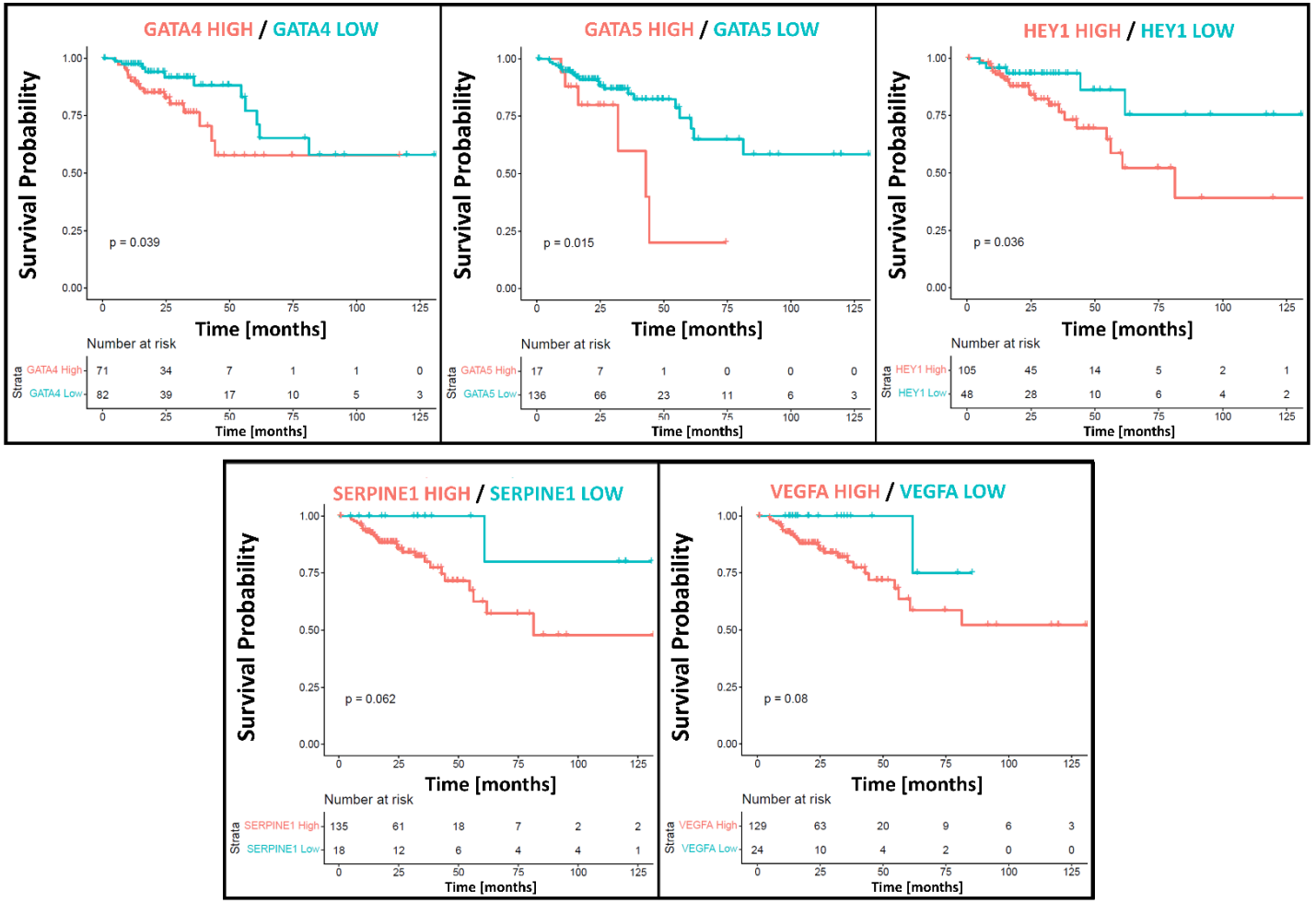

Figure 17. Survival analysis for target genes with significant age or BMI by tissue interaction.

Legend to Figure 17.

The top row shows genes with significant impact on patient survival.

DISCUSSION

Leptin and adiponectin both regulate gene expression of the NOTCH4-GATA4-IRG1 axis in anti-inflammatory macrophages and CC cells. The hormones show distinct differences in affecting gene expression that are associated with tumor progression and patient outcome. Targeted genes showed significant tissue interactions for age and BMI.

Low PPARγ expression is a prognostic factor in CC (232). In the presented coculture model, PPARγ was demonstrated to be downregulated following leptin treatment, but not following adiponectin treatment. This suggests a prognostically relevant tumor promoting role of leptin in CC. When comparing leptin and adiponectin treatment in coculture, diametric cellular responses were also demonstrated for the transcription factor NFκB. While adiponectin induced upregulation of NFκB in both macrophages and CC cells, leptin upregulated NFκB in M2-like macrophages and led to downregulation in CC cells. This suggests that both leptin and adiponectin affect inflammatory mechanisms through NFκB and both can potentially exert carcinogenic effects in TAMs by promoting NFκB overexpression, which regulates CC progression (228, 270).

SERPINE1 expression was slightly upregulated in HT-29 cells following adiponectin treatment, while leptin induced downregulation of SERPINE1 in both cell types. SERPINE1 expression has been demonstrated to be elevated in CRC tissue versus normal colon and associated with disease progression and

recurrence (270). Both obesity-related hormones affect its expression, with adiponectin exerting a potential tumor promoting role.

Upregulation of proinflammatory CCL18 enhances recruitment of regulatory T cells within the colon, which in turn establishes an immunosuppressive environment promoting cancer progression (281). While leptin downregulated CCL18 expression in macrophages, adiponectin induced an upregulation. This could point out another potential carcinogenic role of adiponectin during later stages in CRC, where an anti-inflammatory environment promotes tumor progression (18).

The targeted pro- and anti-inflammatory cytokines that potentially mediate tumor promoting effects were upregulated following adiponectin treatment, except for CCL22 in CC cells. Both obesity-related hormones induced a profound upregulation of IL-6 and IL-8 expression, which both play an essential role in CRC progression (282, 283). IL-6 and IL-1β expression was upregulated by leptin to a much greater degree than by adiponectin; however, both hormones induced an upregulation of these tumor promoting proinflammatory cytokines (275, 283).

RNA-seq analysis revealed significant differential gene expression of 13 target genes between CC and normal colon tissue. Six genes showed significant age or obesity by tissue interactions. No differences were found for the target gene IRG1. The low read counts of this gene among all patient samples suggests that IRG1 has a low expression profile in general. Differences in expression might be masked dependent on the sensitivity (read depth) of the

respective RNA-seq method used to obtain data. Therefore, no clear conclusions concerning IRG1 expression can be drawn.

All target genes of the NOTCH4-GATA4-IRG1 axis that showed a significant age or obesity by tissue interaction were upregulated in young patients or in obese patients, respectively. Furthermore, high expression of GATA4, GATA5 and HEY1 were associated with decreased overall patient survival. This suggests that these genes are factors for poor overall survival in young and obese patients, making them a potential treatment target in EOCC.

CHAPTER X

CONCLUSIONS AND OVERVIEW

Obesity and metabolic dysfunction are strong risk factors of CC and their role in EOCC requires further study. The rising incidence of EOCC as a common cause of cancer death among the young requires investigation of underlying mechanisms and identification of age- and obesity-related target genes for specific treatment strategies.

The 14-day cell line model for differentiating and polarizing THP-1 monocytes produces a distinct anti-inflammatory macrophage phenotype mimicking TAMs. The cell treatment protocol is reproducible and creates an M2-like macrophage phenotype to be studied for underlying inflammatory mechanisms in CC. This is confirmed by upregulation of M2-like anti-inflammatory and tumor-related macrophage markers, such as CD206 or MMP2. These markers play a role in tumor progression and metastasis and are also associated with patient outcome.

This thesis focuses on the obesity-related hormones leptin and adiponectin and the macrophage-specific metabolite itaconate mediating tumor-promoting mechanisms through macrophages by altering gene expression of the NOTCH4-GATA4-IGR1 axis. The data presented herein demonstrate that IRG1

is amplified in sporadic colon adenocarcinoma and that colon tissue concentrations of the ACOD1 protein and the metabolite itaconate differ between age groups. These findings have not yet been reported in CC and provide new insight into its onset and progression. Itaconate may have a particular immunotherapeutic role in patients with CC and obesity, specifically with EOCC. Further studies analyzing larger sample sizes are needed to investigate itaconate in CC and related mechanisms. Itaconate may serve as a potential therapeutic target in patients with EOCC.

Itaconate affects PPARγ gene expression in M2-like macrophages and promotes anti-inflammatory cytokine expression in M2-like macrophages. The obesity-related hormones leptin and adiponectin can induce IRG1 expression and therefore itaconate production and promote tumor promoting marker expression in M2-like macrophages and CC cells in vitro. In contrast, M0 macrophages showed a very limited response to treatment with leptin, adiponectin or itaconate. This suggests that the carcinogenic effects of obesity-related inflammatory factors may play a role in tumor progression, when M2-like TAMs are already established, rather than at tumor onset.

The investigated marker expression patterns in macrophages and CC cells are associated with worse clinical outcomes in patients with CRC. Upregulation of the IRG1-related transcription factor GATA4 in CC is age-dependent, increased in the young and associated with decreased patient survival. Increased expression of the PPARγ-related transcription factor GATA5 is dependent on BMI and is associated with decreased overall survival in patients

with CC. GATA5 expression in CC cells in vitro is upregulated due to leptin treatment in the presence of macrophages. High leptin levels in obese patients with CC may induce tumor-promoting mechanisms through the PPARγ pathway, increasing GATA5 expression.

The NOTCH4-GATA4-IRG1 axis may partly explain the increased incidence in EOCC and may present a new potential therapeutic target in young patients with EOCC. Its target genes provide a link between metabolic dysfunction in obese patients and EOCC. The proinflammatory role of leptin and the anti-inflammatory role of adiponectin that has been postulated in the literature do not concur with the inflammatory cellular responses observed in our coculture model. The effects of obesity-related hormones may also change during cancer development, as this represents a dynamic setting. Further studies are necessary to demonstrate the pathway mechanisms of itaconate that directly mediate carcinogenic effects.

Future Directions

Carcinogenic itaconate production plays a role in colon cancer and macrophage-specific IRG1 expression is upregulated by obesity-related hormones. This suggests a tumor promoting role of leptin and adiponectin through macrophages, which should be validated by functional in vitro experiments in the future. Therefore, macrophage-responses using the cell line model introduced in this thesis should be characterized in more depth. Also proinflammatory M1-like macrophages should be analyzed in this setting.

Since the dichotomous model of M1-like and M2-like macrophages does seem to fully reflect macrophage function, the analysis of gene protein expression, protein and metabolite levels related to macrophage metabolism are important foci for future investigations. Multiplex flow cytometry targeting a wider spectrum of markers can help defining the phenotypic profile of macrophages more accurately. Mitochondrial respiration and glycolysis of macrophages can be quantified using Seahorse Real-Time cell metabolic analysis, and Cytometry by time of flight (CyTOF) measuring multiple metabolites can contribute to a high-dimensional phenotypic and functional profile of the macrophage of interest. Furthermore, the precise definition of the role of itaconate and obesity-related hormones in CC requires a larger sample size of patients providing cancer and normal colon tissue as well as blood. A consecutive patient collective evenly representing different age and BMI groups is of high interest to be able to draw further conclusions on itaconate and obesity-related mechanisms in CC.

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APPENDIX

Macrophage differentiation and polarization into an M2-like phenotype using a human monocyte-like THP-1 leukemia cell line

Protocol

An overview of the steps described in this protocol is shown in **Figure 18**.

The human monocyte-like leukemia cell line THP-1 was purchased from the American Type Culture Collection (ATCC TIB202). We authenticated the THP-1 cell line using short tandem repeat analysis (ATCC).

All steps should be performed under sterile conditions.

The THP-1 monocytic cell line grows in suspension and does not attach to cell culture surfaces. Adherence can be induced by differentiating monocytes into macrophage-like cells through e. g. mechanical stress or specific treatment with *PMA.*

1. Culturing and maintenance of THP-1 monocyte-like cells

1.1 Set a timer for 2 minutes and 30 seconds. Remove the frozen vial containing the THP-1 cell line (**Table 20**) from the liquid nitrogen and thaw it immediately in

a clean water bath (37 °C). Start the timer as soon as the vial is put into the water bath. The cap can be loosened to release pressure that is building up due to the thawing process, but the tube opening should not get in contact with the water to avoid contamination. The optimal time period for thawing the cells lies between 2 minutes and 2 minutes and 30 seconds. Continue thawing the cell suspension until an ice chip of the size of about 4 mm is left within the vial, then proceed to the next step immediately.

1.2 Transfer the liquid phase of the cell suspension to a 15 ml tube containing 9 ml of warm (37 °C) growth media (**Table 20**). Then transfer 1 ml of the warm medium-cell-suspension into the THP-1 vial and back into the 15 ml tube to melt the remaining ice chip and to flush the vial. This step assures that no cells are left behind.

1.3 Mix the suspension gently by pipetting up and down with a 1000 µl pipette. Remove a small sample (approximately 10 µl) to count the cells for viability (using trypan blue for exclusion) while they are spun. Spin down the warm cell suspension at 200 x g for 7 minutes at 37 °C.

1.4 Remove the supernatant completely and resuspend with a certain volume of warm growth medium to achieve a cell density of $5x10^5$/ml. Mix your suspension gently and transfer 22 ml of volume into T-75 cell culture flasks (**Table 20**). Store

flasks upright in an incubator at 37 °C with 5 % carbon dioxide (CO_2) concentration. Exchange growth media every 3-4 days.

2. Seeding of THP-1 cells and differentiation into M0 macrophages

2.1 Prepare the cell containing growth medium with the respective cell density to seed cells at a density of $3x10^5$/ml/well into 24-well cell culture plates (**Table 20**). Mix the medium gently and prepare aliquots of 26 ml, each put into a 50 ml-tube. Use each 26 ml-aliquot for seeding the cells into a respective plate.

2.2 Transfer 1ml of cell-containing medium into each well of a 24-well plate. Mix the media gently by pipetting up and down between transfers.

2.3 Prepare a stock solution of PMA (1 mg PMA dissolved in 100 µl of Dimethyl Sulfoxide (DMSO) = ~16 mM solution of PMA in DMSO) and dilute it with cold Phosphate Buffered Saline (PBS) to a final working concentration of 10 ng/µl right before cell treatment (**Table 20**). Keep the solution on ice and use it immediately. Do not refreeze.

Add 100 ng of PMA per well. Let each cell plate sit in the incubator without any further treatment for 72 hours.

2.4 After 72 hours, remove the growth medium and replace it with 1 ml of fresh growth medium. Do not touch the bottom of the wells with pipette tips. Let cells rest for another 96 hours in the incubator.

2.5 After 96 hours, repeat step 2.4 (media change) and let the cells rest for another 24 hours.

The M0 macrophages are now ready to be used for experiments (**Figure 19**). Immediately prior to treating the cells as part of further experiments, consider a media change with RPMI only (**Table 20**), since growth media supplements can cause interference with reagents that are added for cell treatment.
In case M2-like macrophages are needed, proceed with section 3.

3. Polarization of M0 macrophages into M2-like macrophages

3.1 Prepare a stock solution of IL-4 and IL-13 (20 µg IL-4 or IL-13 dissolved in 200 µl nuclease-free water, respectively) and dilute it to a final working concentration of 2 ng/µl with PBS immediately prior to cell treatment. Keep the solution on ice and use it immediately. Do not refreeze.
Remove the growth medium and replace it with 1 ml of fresh growth medium. Add 20 ng interleukin 4 (IL-4) and 20 ng interleukin 13 (IL-13) per well. Let cells rest for 48 hours in the incubator.

3.2 After 48 hours, repeat step 3.1. Let cells rest for another 48 hours in the incubator.

3.3 Remove the growth medium and replace it with 1 ml of fresh growth medium. Let cells rest for 48 hours in the incubator.

M2-like macrophages are now ready to be used for experiments (**Figure 19**). Immediately prior to treating the cells as part of further experiments, consider a media change with RPMI only (**Table 20**), since growth media supplements can cause interference.

4. Detaching and harvesting macrophages for flow cytometry

To detach and harvest polarized macrophages from plates for flow cytometry, a mechanical method combining cold shocking and cell scraping is used.

4.1 Remove warm cell medium and replace it with a mixture of ice-cold PBS (without calcium and magnesium) and 5 % fetal bovine serum (FBS), 1 ml per well. Immediately after this, place the cell plate on ice for 45 minutes. Do not place the cell plate on ice before the warm cell medium is removed, since this will decrease cell viability significantly. Keep cells on ice only once a cold shock with ice-cold PBS/5 % FBS mixture is induced.

4.2 After 45 minutes on ice, scrape off cells using mini cell scrapers (**Table 20**). Gently transfer the detached macrophages in cold PBS/5 % FBS into a 15 ml tube. Keep the tube on ice at all times until cells are stained.

Note: To reach adequate cell counts for staining, we recommend pooling eight wells of cells together

Table 20. Table of materials.

Name of Material/ Equipment	Company	Catalog Number
0.4% trypan blue	VWR	152-5061
1.5ml microcentrifuge tube	USA Scientific	1615-5510
1000µl TipOne pipet tips	USA Scientific	1111-2821
10ml serological pipet	VWR	89130-898
15ml Centrifuge tube	VWR	89039-664
200µl TipOne pipet tips	USA Scientific	1120-8810
20µl TipOne pipet tips	USA Scientific	1120-1810
25ml serological pipet	VWR	89130-900
50ml Centrifuge tube	VWR	89039-662
5ml serological pipet	VWR	89130-896
Antibiotic Antimycotic Solution (100x), stabilized	Sigma	A5955-100ml
Binder CO2 Incubator	VWR	C170-ULE3
CytoOne T-75cm flask with filter cap	USA Scientific	CC7682-4875
Dulbecco's Phosphate Buffered Saline (PBS)	Sigma	D8537-500ml
Eppendorf Centrifuge 5804 R (refrigerated)	Eppendorf	-
Ethyl alcohol (70%)	-	-
FACSCalibur flow cytometer	BD Biosciences	-
Falcon 24-well plate	VWR	353504
Fetal Bovine Serum (FBS)	ATCC	30-2020
FITC Mouse Anti-Human CD14	BD Biosciences	555397
FITC Mouse Anti-Human CD80	BD Pharmingen	557226
FITC Mouse IgG1 κ Isotype Control	BD Pharmingen	555748
FITC Mouse IgG2a, κ Isotype Control	BD Biosciences	553456
Human BD Fc Block	BD Biosciences	564220
Human interleukin 13 (IL-13)	R&D	IL-771-10ug
Human interleukin 4 (IL-4)	R&D	SRP3093-20ug
Labconco Biosafety Cabinet (Delta Series 36212/36213)	Labconco	-
L-Glutamine Solution, 200 mM	ATCC	30-2214
Lipopolysaccharide (LPS) from E. coli 0111:B4	Sigma	L2630-100mg
Mini Cell Scrapers	Biotium	22003

Neubauer hemocytometer	Fisher Scientific	02-671-5
Nikon Eclipse inverted microscope TS100	Nikon	-
Nuclease-free water	Invitrogen	AM9937
Olympus Light Microscope RH-2	Microscope Central	40888
P10 variable pipet- Gilson	VWR	76180-014
P1000 variable pipet-Gilson	VWR	76177-990
P200 variable pipet- Gilson	VWR	76177-988
PE Mouse Anti-Human CD11b	BD Biosciences	555388
PE Mouse IgG1, κ Isotype Control	BD Biosciences	555749
PE-Cy 5 Mouse Anti-Human CD206	BD Pharmingen	551136
PE-Cy 5 Mouse IgG1 κ Isotype Control	BD Pharmingen	555750
Phorbol 12-myristate 13-acetate (PMA)	Sigma	P8139
Powerpette Plus pipettor	VWR	75856-448
Precision Water bath (model 183)	Precision Scientific	66551
RPMI-1640 Medium	ATCC	30-2001
THP-1 cell line, American Type Culture Collection (ATCC)	ATCC	TIB-202

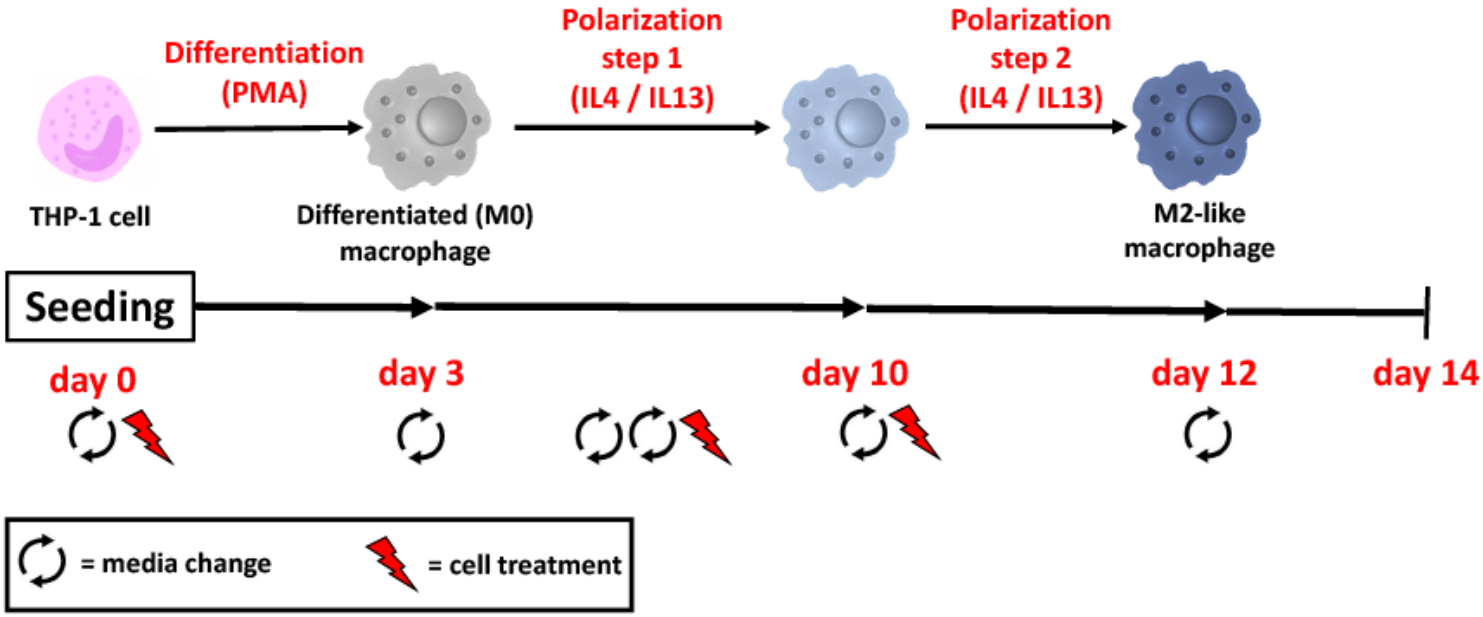

Figure 18. Protocol overview of the M2-like macrophage cell line model.

Legend to Figure 18.

On day 0, cells are seeded into plates with a growth medium and incubated with PMA for 72 h. On day 3 and day 7 cell medium is changed, which lets the cells rest without PMA for a total of 120 h. On day 8, the growth medium is changed once more, and cells are incubated with IL-4 and IL-13 to induce M2-like polarization. This step is repeated after 2 days, on day 10. On day 12, the last medium change is performed, and M2-like cells rest in the growth medium for another 48 h before being used for experiments (PMA = phorbol 12-myristate-13-acetate; IL = interleukin).

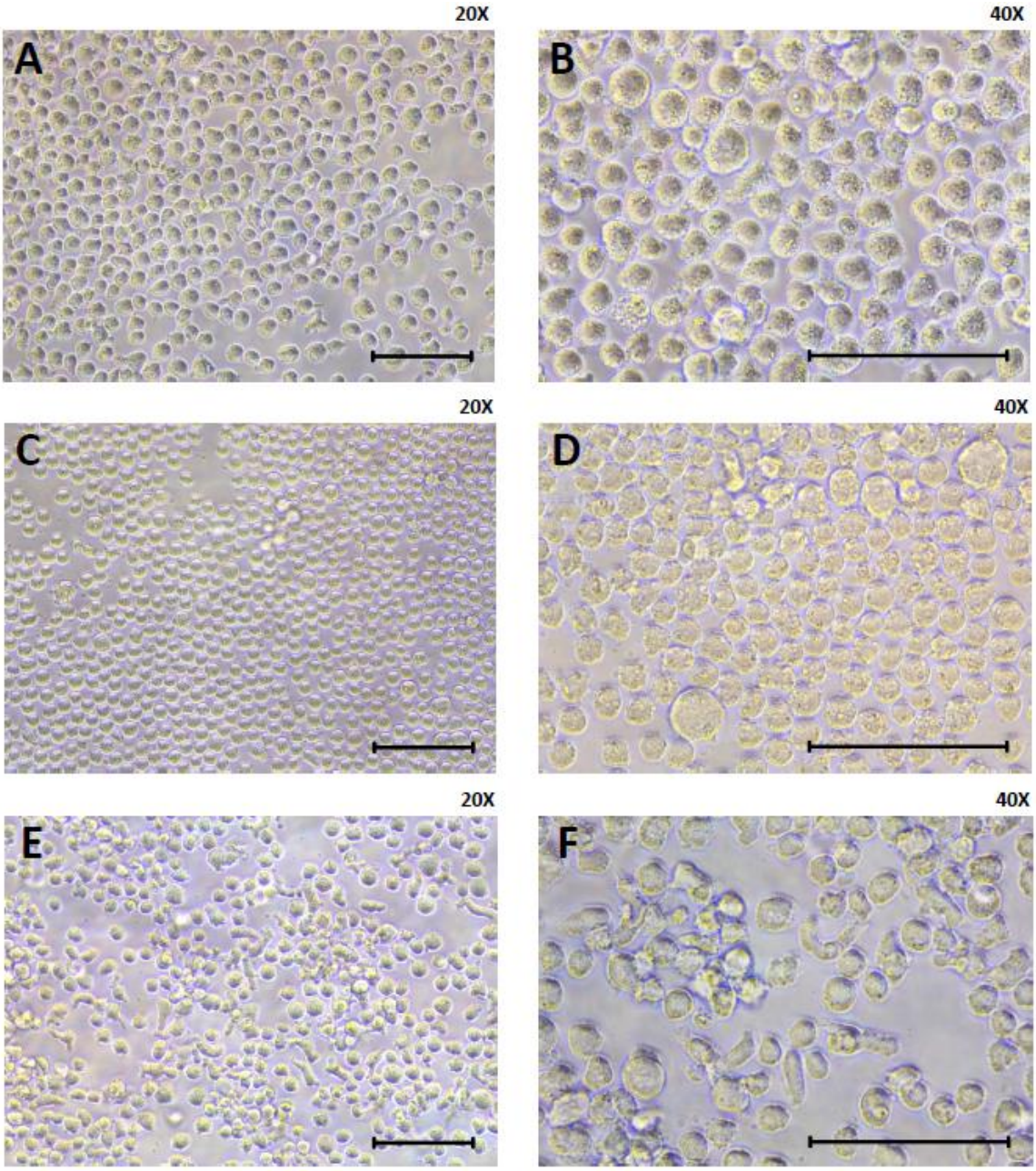

Figure 19. Cell morphology of THP-1 cells differentiated (M0) macrophages, and M2-like macrophages using light microscopy.

Legend to Figure 19.

Cells were seeded at 3 x 105 per well in a 24-well plate. (**A**,**B**) THP-1 cells are shown at baseline. (**C**,**D**) The
differentiated M0 macrophages received PMA treatment for 72 h, growth medium change and a 96-h-resting period. (**E**,**F**)
The M2-like macrophages are shown after completed polarization treatment with IL-4 and Il-13 at day 14 of this cell model
(20x and 40x magnification; scale = 100 µm).

CURRICULUM VITAE

Katharina M. Scheurlen, MD

Address:	642 Baxter Ave, Louisville, KY 40204
Date of birth:	June 24, 1989
Place of birth:	Heidelberg, Germany
Phone:	+1 502 551 9079
E-mail:	Katharina.scheurlen@louisville.edu

Education

1999 - 2008	Comprehensive School Mutterstadt, Germany
2008	Abitur
2008 - 2015	University of Heidelberg, Medical School, Germany
since 08/2019	PhD program, Department of Physiology, University of Louisville School of Medicine, Louisville, Kentucky

Surgical training

2014 - 2015	Practical year as medical student
since 11/2015	Surgical residency, Department of Surgery, University of Heidelberg, (M. Büchler, M.D.); research work at the Turhan laboratory for Metabolic Surgery, Section of Minimally Invasive and Metabolic Surgery, Department of Surgery, University of Heidelberg
since 08/2019	Price Fellow of Surgical Research, Department of Surgery, University of Louisville, Kentucky, USA, (S. Galandiuk, M.D.)

Academic appointments

06/2015	M.D. Thesis: University of Heidelberg, Germany: “Stellenwert der Galaktographie zur Abklaerung pathologischer Sekretion in der komplementaeren Mammadiagnostik“ (Mentor: K. Wasser, M.D. / S. O. Schoenberg, M.D., Ph.D.)
05/2022	Ph.D. Thesis: Department of Physiology and Biophysics, School of Medicine, University of Louisville, Kentucky, United States of America. “The Role of Obesity in Macrophage-mediated Mechanisms promoting early-onset Colon cancer" (Mentor: Susan Galandiuk, M.D.)

International presentations

2017	German Society of Surgery (DGCH) in Munich, Germany, oral presentation on “Microvascular complications in patients with type 2 diabetes mellitus – metabolic surgery vs. medical treatment: a systematic review and meta-analysis”
2017	International Federation Surgery of Obesity and Metabolic Disorders (IFSO) in London, United Kingdom, poster presentation on “Metabolic surgery improves diabetic nephropathy independent of weight loss: a systematic review with meta-analysis”
2018	International Federation for the Surgery of Obesity and Metabolic Disorders – European Chapter (IFSO-EC) in Athens, Greece, oral presentation on “Serum uromodulin in diabetic nephropathy - early diagnosis using serum markers”
2021	Central Surgical Association (CSA) in Chicago, USA, oral presentation on “Early-onset colorectal cancer – new insights on macrophage function, itaconate and obesity”

Grants/Funding

03/2017	Heidelberg Surgery Foundation: 12.000€
03/2018	Heidelberg Surgery Foundation: 10.000€

05/2018	Mentorship program *Research Talent Academy*, European Association for Endoscopic Surgery (*EAES*), Chairman Dr. Sheraz Markar, London
03/2019	Heidelberg Surgery Foundation: 10.000€

Reviewer activities

- Diseases of the Colon and Rectum
- Langenbeck's Archives of Surgery

Supervisory and Teaching Activity

- Instructor for surgical nurses and medical students at the University of Heidelberg

Societies

- Member of the American Society of Colon and Rectal Surgeons (ASCRS)

Research

- **Scheurlen KM**, Schnitzer A, Krammer J, Kaiser C, Schonberg SO, Wasser K. Value of galactography for the diagnostic work-up of pathological nipple discharge in multimodal breast diagnostics. Part 1: An online survey among German breast care centers. Radiologe. 2014;54:63–67.

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- **Scheurlen KM**, Snook DL, Littlefield A, George J, Riggs DW, Beal RJ, Gaskins J, Galandiuk S. Anti-inflammatory mechanisms in cancer research: a distinct M2-like macrophage model derived from the THP-1 cell line.
  Submitted to PLOS ONE, February 2022

Book Chapter

- **Scheurlen KM**, Kavalukas SL, Galandiuk S.
  Diverticular Disease of the Colon.
  Cameron & Cameron, 14th Edition