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TLEMCEN UNIVERSITY - ABOU-BEKRBELKAÏD
SNV- STU FACULTY
BIOLOGY DEPARTMENT
LABORATORY OF APPLIED MOLECULAR BIOLOGY AND IMMUNOLOGY,
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THESIS

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Specialty: Applied Immunology
By:

Nihel Chahinez DJEBRI spouse of KORSO FECIANE

Title:

Metformin Effects on Lipid Droplet Formation and NLRP3/NF-κB Activation in Monocytes during Colorectal Cancer

Jury:

Pr. Hafida HASSAINE	University of Tlemcen, Algeria	President
Pr. Mourad ARIBI	University of Tlemcen, Algeria	Dir. thesis
Pr. Houda BELGUENDOUZ	USTHB, Algeria	Examinator
Pr. Franck MENNECHET	University of Montpellier, France	Examinator
Pr. Mohammed chems-eddine SMAHI	University of Tlemcen, Algeria	Examinator

Résumé

Introduction : Le cancer colorectal (CRC) génère un microenvironnement tumoral (TME) complexe, connu pour altérer profondément la fonction et le phénotype des cellules immunitaires, y compris les monocytes. Les aspects clés de cet environnement peuvent être reproduits par le milieu conditionné tumoral (TCM). La metformine a émergé comme un candidat prometteur pour contrer la dérégulation immunitaire et inflammatoire induite par le TCM. Par conséquent, cette étude vise à évaluer les effets de la metformine sur l'expression de l'inflammasome NOD-like receptor family pyrin domain containing 3 (NLRP3), la distribution des sous-populations des monocytes et l'accumulation de gouttelettes lipidiques (LD) lors de l'exposition au TCM colorectal.

Matériels et méthodes : Les essais ont été réalisés sur des monocytes humains primaires exposés au TCM colorectal en présence ou en absence de metformine.

Résultats : Le TCM a significativement augmenté la production d'oxyde nitrique (NO, $p < 0,001$) et de peroxyde d'hydrogène (H_2O_2, $p < 0,001$), les niveaux d'ions de calcium intracellulaires libres ($_{if}Ca^{2+)}$ ($p < 0,001$), l'expression du facteur nucléaire kappa-light-chain-enhancer des cellules B (NF-κB p65/RelA, $p < 0,001$), de NLRP3 ($p < 0,01$) et de l'interleukine-1 bêta (IL-1β, $p < 0,0001$), ainsi que les triglycérides intracellulaires ($_{i}$TAGs, $p < 0,05$), le contenu cellulaire total en cholestérol ($_{tcc}$CHOL, $p < 0,001$) et l'accumulation de gouttelettes lipidiques ($p < 0,05$). Il a également altéré l'activité phagocytaire ($p < 0,05$) et modifié le phénotype des monocytes, avec une orientation vers un phénotype CD14low, CD16high ($p < 0,0001$ pour les deux marqueurs). Notamment, le traitement par la metformine a exercé des effets inverses larges et significatifs, en particulier sur l'explosion respiratoire (NO, $p < 0,05$; H_2O_2, $p < 0,01$), les niveaux de $_{if}Ca^{2+}$ ($p < 0,01$), l'expression de NF-κB p65/RelA et de NLRP3, ainsi que la production d'IL-1β ($p < 0,0001$ pour tous), et l'accumulation de gouttelettes lipidiques ($p < 0,0001$), des $_{i}$TAGs ($p < 0,001$) et du $_{tcc}$CHOL ($p < 0,01$). La metformine a également restauré de façon significative l'expression de CD14 ($p < 0,05$) et augmenté le taux des monocytes CD14+ ($p < 0,01$), tout en réduisant l'expression de CD16 ($p < 0,05$) et la fréquence des monocytes CD16+ ($p < 0,01$). Cependant, elle n'a eu aucun effet significatif sur la phagocytose des monocytes exposés au TCM ($p > 0,05$).

Conclusion : Nos résultats démontrent que la metformine exerce une action ciblée de reprogrammation immunométabolique sur les monocytes exposés aux signaux tumoraux colorectaux. Ces données soutiennent son intérêt en tant qu'agent immunomodulateur spécifique du contexte tumoral et ouvrent la voie à de futures recherches translationnelles visant à évaluer son utilisation comme agent adjuvant dans les cancers associés à l'inflammation.

Mots clés : Cancer colorectal ; NF-Kb p65/RelA ; NLRP3 inflammasome ; Gouttelettes lipidiques ; Metformine ; Microenvironnement tumoral ; Monocytes ; Milieu conditionné tumoral.

Abstract

Background: Colorectal cancer (CRC) generates a complex tumor microenvironment (TME) known to profoundly alter the function and phenotype of immune cells, including monocytes. Key aspects of this environment can be mimicked by tumor-conditioned medium (TCM). Metformin has emerged as a promising candidate to counteract TCM-induced immune and inflammatory dysregulation. Therefore, this study aimed to evaluate the effects of metformin on NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome expression, monocyte subset distribution, and lipid droplet (LD) accumulation upon exposure to colorectal TCM.

Methods: Assays were performed on primary human monocytes exposed to colorectal TCM in the presence or absence of metformin.

Results: TCM significantly increased nitric oxide (NO, $p < 0.001$) and hydrogen peroxide (H_2O_2, $p < 0.001$) production, intracellular free calcium ions ($_{if}Ca^{2+}$) ($p < 0.001$) levels, expression of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB p65/RelA, $p < 0.001$), NLRP3 ($p < 0.01$), and interleukin-1 beta (IL- 1β, $p < 0.0001$), as well as intracellular triacylglycerols ($_{i}$TAGs, $p < 0.05$), total cellular cholesterol content ($_{tcc}$CHOL, $p < 0.001$), and lipid droplet accumulation ($p < 0.05$). It also impaired phagocytic activity ($p < 0.05$) and altered monocyte phenotype, along with a shift toward a CD14low, CD16high phenotype ($p < 0.0001$ for both markers). Notably, metformin treatment exerted broad and significant reversing effects, specifically on respiratory burst (NO, $p < 0.05$; H_2O_2, $p < 0.01$), $_{if}$Ca2+ levels ($p < 0.01$), NF-κB p65/RelA and NLRP3 expression, and IL-1β production ($p < 0.0001$ for all), and the lipid droplet accumulation ($p < 0.0001$), $_{i}$TAGs ($p < 0.001$), and $_{tcc}$CHOL ($p < 0.01$). Metformin also significantly restored CD14 expression ($p < 0.05$) and increased CD14+ monocyte frequency ($p < 0.01$), while reducing CD16 expression ($p < 0.05$) and CD16+ monocyte frequency ($p < 0.01$). However, it had no significant effect on phagocytosis in TCM-exposed monocytes ($p > 0.05$).

Conclusions: Our findings highlight metformin's selective immunometabolic reprogramming capacity in monocytes exposed to colorectal tumor-derived signals, supporting its potential as a context-specific immunomodulator. This study lays the groundwork for future translational research on metformin as an adjunctive agent in inflammation-driven tumor settings.

Keywords: Colorectal cancer ; NF-κB p65/RelA ; NLRP3 inflammasome ; Lipid droplets ; Metformin ; Monocyte subsets ; Tumor microenvironment ; Tumor-conditioned medium.

ملخص

مقدمة :يُنتج سرطان القولون والمستقيم (CRC) بيئة دقيقة معقدة تُعرف ببيئة الورم الدقيقة(TME) ، والتي تؤثر بشكل عميق على وظيفة وخاصيات الخلايا المناعية، بما في ذلك الوحيدات. ويمكن محاكاة الجوانب الرئيسية لهذه البيئة بواسطة الوسط المشروط بالورم (TCM) وقد برزت الميتفورمين كخيار واعد لمواجهة الاضطرابات المناعية والالتهابية التي يسببها الـ TCM لذلك، هدفت هذه الدراسة إلى تقييم تأثير الميتفورمين على تعبير الجسيم الملتهب NOD-like receptor family pyrin domain containing 3 (NLRP3)، وتوزيع الأنماط الفرعية للوحيدات، وتراكم القطرات الدهنية (LD)عند التعرض للـ TCM المشتق من سرطان القولون والمستقيم.

الوسائل والطرق :أُجريت التجارب على وحيدات بشرية أولية عُرضت للـ TCM القولوني والمستقيمي في وجود أو غياب الميتفورمين.

النتائج :أدى الـ TCM إلى زيادة ملحوظة في إنتاج أوكسيد النتريك (NO, $p < 0.001$) وبيروكسيد الهيدروجين (H_2O_2, $p < 0.001$)، ومستويات الكالسيوم الحر داخل الخلية($_{if}Ca^{2+}$, $p < 0.001$) ، وتعبير عامل النسخ NF-κB p65/RelA ($p < 0.001$)، و NLRP3 ($p < 0.01$) ، والإنترلوكين-1 بيتا .(IL-1β, $p < 0.0001$) كما تسبب في زيادة الدهون الثلاثية داخل الخلايا($_{i}TAGs$, $p < 0.05$) ، وإجمالي محتوى الكوليسترول الخلوي ($_{tcc}CHOL$, p < 0.001)، وتراكم القطرات الدهنية .($p < 0.05$) كذلك قلل من النشاط البلعمي ($p < 0.05$) وأحدث تحولاً في نمط الوحيدات نحو النمط CD14^low, CD16^high ($p < 0.0001$) لكل من الواسمين). بشكل لافت، أظهر علاج الميتفورمين تأثيرات عكسية واسعة ومهمة، خصوصاً على الانفجار التنفسي(NO, $p < 0.05$؛ H2O2, $p < 0.01$) ، ومستويات($_{if}Ca^{2+}$ $p < 0.01$) ، وتعبير NF-κB p65/RelA وNLRP3 ، وإنتاج IL-1β ($p < 0.0001$) لجميعها)، بالإضافة إلى تراكم القطرات الدهنية($p < 0.0001$) ، والدهون الثلاثية داخل الخلايا($_{i}TAGs$, $p < 0.001$) ، والكوليسترول الكلي الخلوي .($_{tcc}CHOL$, $p < 0.01$) كما أعاد الميتفورمين بشكل ملحوظ التعبير عن CD14 ($p < 0.05$)وزاد من نسبة الوحيدات($p < 0.01$) CD14+ ، بينما خفض تعبير CD16 ($p < 0.05$) وتواتر الوحيدات CD16+ ($p < 0.01$).ومع ذلك، لم يكن له تأثير كبير على البلعمة في الوحيدات المعرضة للـ TCM ($p > 0.05$).

الاستنتاجات :تُبرز نتائجنا قدرة الميتفورمين على إعادة برمجة المناعة والتمثيل الغذائي بشكل انتقائي في الوحيدات المعرضة للإشارات المشتقة من الورم القولوني والمستقيمي، مما يدعم دوره كمنظم مناعي نوعي في سياق الأورام. وتضع هذه الدراسة الأساس لأبحاث مستقبلية تطبيقية حول الميتفورمين كعامل مساعد في البيئات الورمية المرتبطة بالالتهاب.

الكلمات المفتاحية :سرطان القولون والمستقيم ; العامل النووي كابا-بي p65/RelA ; الجسيم الملتهب NLRP3 ; القطرات الدهنية ;الميتفورمين ;الأنماط الفرعية للوحيدات ;البيئة الدقيقة للورم ;الوسط المشروط بالورم.

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Dedications

"*They are no longer where they were, but they are everywhere I am*."

— Victor Hugo

In loving memory of my dear parents, who left too soon…You will forever remain alive in my heart and in every one of my achievements. This work is the fruit of your sacrifices, your love, and your silent prayers. May Allah grant you His infinite mercy and open for you the gates of His Paradise.

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Nihel Chahinez DJEBRI

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LIST OF ABREVIATIONS

A

ACAT: *acyl-CoA : cholesterol acyl-transferase*

ADCC: *Antibody-Dependent Cellular Cytotoxicity*

AGE: *Advanced Glycation End-products*

AKT: *protein kinase B*

AMPK: *AMP-activated protein kinase*

APC: *Adenomatous Polyposis Coli*

ASC: *apoptosis-associated speck-like protein*

ATP: *adenosine triphosphate*

B

BCL-2: *B-cell lymphoma 2*

BMI: *Body Mass Index*

BRAF: *v-raf murine sarcoma viral oncogene homolog B*

C

CIMP: *CpG Island Methylator Phenotype*

CGI: *CpG Island*

CCL2: *chemokine (C-C motif) ligand 2* (or **MCP-1**, monocyte chemoattractant protein-1).

CCR2: *C-C chemokine receptor type 2*

CCR5: *C-C chemokine receptor type 5*.

CD14: *cluster of differentiation 14*

CD16: *cluster of differentiation 16* (FcγRIII).

CD36: *cluster of differentiation 36*

CD47: *cluster of differentiation 47* (« do-not-eat-me » signal).

CD: *Crohn's disease*

CDK: *Cyclin-dependent kinase*

CTFC: *corrected total cell fluorescence*

CRC: *Colorectal Cancer*

CCR: *Cancer Colorectal*

CIN: *Chromosomal Instability*

COX-2: *cyclo-oxygenase 2*

D

DAMPs: *Damage-Associated Molecular Patterns*

DCs: *Dendtritic cells*

dMMR: *deficient Mismatch Repair*

DGAT: *Diacylglycerol acyltransferase*

DNA: *Deoxyribonucleic Acid*

DT2: T*ype 2* diabetes

E

ECM: *Extracellular matrix*

EV: *Extracellular Vesicles*

ER: *Endoplasmic Reticulum*

ERK: *Extracellular signal-Regulated Kinase*

F

FBS: *Foetal bovin serum*

Fas/FasL: *Fas receptor / Fas ligand*.

FcγR: *Fc gamma receptor*

FcγRIII (CD16): *A subtype of the Fc receptor for immunoglobulin G (IgG)*

FATP: *Fatty Acid Transport Protein*

G

GSDMD: *Gasdermin D* pyroptosis protein

GLUT1: *Glucose Transporter type 1*

H

H_2O_2: *Hydrogen peroxide*

HIF-1α: *Hypoxia-Inducible Factor 1 alpha*

HMGCR: *3-Hydroxy-3-Methylglutaryl-CoA Reductase*

I

IBD: *Inflammatory Bowel Diseases*

ICAM: *Intercellular Adhesion Molecule*.

IFN-γ: *Interferon gamma*

$_{if}Ca^{2+}$: *Intracellular free calcium ions*

IGF-1: *Insulin-like Growth Factor 1*

IL: *Interleukin*

IL-1β: *Interleukin-1 beta*.

IL-6: *Interleukin-6*.

IL-10: *Interleukin-10*.

IL-12: *Interleukin-12*.

IL-18: *Interleukin-18*.

IR: *Insulin Receptor*

IRS2: *Insulin Receptor Substrate 2*

IκBα: *Inhibitor of kappa B alpha*

IKK: *IκB Kinase*

¡TAG: *intracellular triacylglycerol*

J

JAK/STAT: *Janus Kinase / Signal Transducer and Activator of Transcription*

K

KRAS: *Kirsten rat sarcoma viral oncogene homolog*

L

LD: *Lipid Droplet*

LDLR: *Low-Density Lipoprotein Receptor*

LDL: *Low-Density Lipoprotein*

Lgr5: *Leucine-rich repeat-containing G-protein coupled receptor 5*

LPS: *Lipopolysaccharide*

LRR: *Leucine-rich Repeat*

LXR: *Liver X Receptor*

M

MAPK: *Mitogen-Activated Protein Kinase*

MCP-1: *Monocyte Chemoattractant Protein-1*

M-CSF: *Macrophage Colony-Stimulating Factor*

METF: *Metformin*

MHC / CMH: *Major Histocompatibility Complex*

MLH1: *MutL Homolog 1*

MMR: *Mismatch Repair*

MMP: *Matrix Metalloproteinase*

MSH2: *MutS Homolog 2*

MSH6: *MutS Homolog 6*

MSI: *Microsatellite Instability*

mTOR: *Mammalian Target of Rapamycin*

MyD88: *Myeloid Differentiation Primary Response 88*

N

NACHT: *NAIP, CIITA, HET-E and TP1* domain

NBT: *Nitro-blue tetrazolium*

NFKB / NF-κB: *Nuclear Factor kappa-light-chain-enhancer of activated B cells*

NK: *Natural Killer*.

NKG2D: *Natural Killer Group 2, member D*

NLRP3: *NOD-like Receptor Family, Pyrin Domain Containing 3*

NO: *Nitric Oxide*

NPC1L1: *Niemann-Pick C1-like 1*

O

ORO: *Oil red O*

P

p53: T*umor suppressor protein p53*

PAB: *Peroxide assay buffer*

PAMPs: *Pathogen-Associated Molecular Patterns*

PBMCs: *Peripheral blood mononuclear cells*

PI3K: *Phosphoinositide 3-Kinase*

PMS2: *Postmeiotic Segregation Increased 2*

PRR: *Pattern Recognition Receptor*

PRS: *Phenol Red Solution*

PYD: ***N-terminal pyrin domain***

PYD: *Pyrin Domain*

R

RAGE: *Receptor for Advanced Glycation End-products*

RAF: *Rapidly Accelerated Fibrosarcoma*

RAS: *Rat sarcoma viral oncogene homolog*

Rb: *Retinoblastoma protein*

ROS: *Reactive Oxygen Species*

RNS: *Reactive Nitrogen Species*

S

S6K1: *Ribosomal Protein S6 Kinase Beta-1*

SE: *Sterol esters*

SCFAs: Short chain fatty acid

SM: *Squalene monooxygenase*

SNPs: *Single Nucleotide Polymorphisms*

SOAT / ACAT: *Sterol O-Acyltransferase / Acyl-CoA:cholesterol acyltransferase*

STAT3: *Signal Transducer and Activator of Transcription 3*

SREBPs: *Sterol Regulatory Element-Binding Proteins*

sFasL: *Soluble Fas Ligand*

T

TAG: *Triacylglycerol*

TAMs: *Tumor-Associated Macrophages*

TBET: *Trypan blue exclusion test*

TCM: *Tumor conditioned medium*

$_{tcc}$CHOL: *Total cholesterol content*

TCR: *T* cell *Receptor*

TCS2 / TSC2: *Tuberous Sclerosis Complex 2*

TGF-β: *Transforming Growth Factor-beta*

Th1/Th2: *Subtypes of T helper lymphocytes type 1 and 2*

TLR4: *Toll-Like Receptor 4*

TME: *Tumor microenvironment*

TNF-α: *Tumor Necrosis Factor-alpha*

TRAIL: *TNF-related apoptosis-inducing ligand*

U

UC: *Ulcerative Colitis*

V

VHL: *Von Hippel-Lindau tumor suppressor*

VEGF: *Vascular Endothelial Growth Factor*

Introduction

Introduction

Colorectal cancer ranks among the most prevalent and deadly cancers worldwide [1]. In addition to genetic and epigenetic alterations, its progression is strongly shaped by the tumor microenvironment [2], a complex network of cytokines, chemokines, metabolites, and other soluble mediators released by tumor and stromal cells [3,4]. These factors not only support tumor growth and immune evasion but also exert profound effects on the function and phenotype of surrounding immune cells [5]. Among its key effector cells, monocytes play a central role in pathogen sensing, phagocytosis, and the orchestration of inflammatory responses [6–9]. Their remarkable plasticity allows them to dynamically adapt to environmental cues, particularly within the tumor context. The functional distribution of monocyte subsets, often characterized by CD14 and CD16 expression [10], reflects their activation state and determines whether they adopt proinflammatory or tissue- reparative functions [11].

While the tumor microenvironment is highly complex and shaped by diverse cellular components, it can be partially mimicked by tumor- conditioned media [12], which contains a mixture of cytokines, metabolites, and danger-associated molecular patterns (DAMPs), capable of influencing the behavior and polarization of immune cells, including monocytes. These factors can reprogram monocyte function, notably by modulating intracellular inflammatory mediators such as the NLRP3 inflammasome and the transcription factor NF-κB [13,14]. Of note, certain transformed cancer cells have been reported to influence the metabolic landscape of immune cells, potentially affecting lipid metabolism and storage [15]. However, the extent to which tumor-derived soluble factors modulate lipid droplet accumulation in monocytes re- mains to be fully elucidated.

Introduction

Given the potential of the tumor microenvironment to reprogram immune cell metabolism and function, there is growing interest in pharmacological strategies capable of counteracting these effects are of growing interest. Among them, metabolic modulators such as metformin (1,1- dimethylbiguanide hydrochloride), widely used as a first-line treatment for type 2 diabetes, have garnered attention for their broad actions. Beyond its glucose-lowering effect, metformin has been shown to exert immunomodulatory effects in diverse experimental settings, including cancer [16]. Moreover, it has been reported to influence lipid metabolism and mitigate inflammatory responses [17], positioning it as a promising candidate for modulating immune cell dysfunctions observed in the colorectal cancer microenvironment [18].

In particular, metformin has been shown to interfere with key inflammatory signaling pathways such as NF-κB and the NLRP3 inflammasome, both of which play central roles in chronic inflammation and cancer-related immune dysregulation [19,20]. NF-κB, a nuclear transcription factor, governs the expression of various proinflammatory genes, while NLRP3, a component of the inflammasome complex, pro- motes caspase-1 activation and thereby the maturation and release of interleukin-1β (IL-1β) [21], a key effector cytokine in inflammation and in the immune dysfunction associated with tumors [22,23]. Importantly, recent findings suggest that metformin may suppress the activation of NF-κB through the inhibition of reactive oxygen species (ROS) and the activation of AMP-activated protein kinase (AMPK), thereby indirectly dampening NLRP3 inflammasome signaling [18,24]. However, the direct link between metformin and NLRP3 inflammasome regulation, particularly in primary human monocytes exposed to tumor-derived conditions, remains insufficiently understood and largely unexplored. Addressing this critical gap is the central aim of our study. Building on this

context, the present study investigates the effects of metformin on NLRP3 inflammasome expression, monocyte subset distribution, and lipid droplet accumulation in response to colorectal tumor-conditioned medium.

Chapter1: Bibliographic Review

1.1. Colorectal cancer

1.1.1. Epidemiology

Colorectal cancer (CRC) represents a major global public health burden and remains one of the most commonly diagnosed malignancies worldwide. According to recent estimates, CRC ranks as the third most frequently diagnosed cancer [27] and the second leading cause of cancer-related mortality globally, with more than 1.93 million new cases and 935 000 deaths globally in 2020 [1]. The incidence of CRC continues to rise, particularly in low- and middle-income countries, largely as a consequence of epidemiological transition and the increasing adoption of Westernized lifestyles, including dietary changes, physical inactivity, and obesity [25].

In Algeria, colorectal cancer is among the most frequently diagnosed cancers in adults and represents a growing cause of cancer-related morbidity and mortality. Data derived from regional cancer registries and hospital-based studies indicate a steady increase in CRC incidence over recent decades, predominantly affecting individuals over the age of 50, with a slight male predominance [26].

1.1.2. Histological types

More than 90% of colon tumors are adenocarcinomas and arise from a benign colonic adenoma or polyp [29].

Several other histological types have also been described:

1.1.2.1. Medullary carcinoma

This is a very rare type of cancer, characterized by sheets of regular, highly cohesive cells in which no glandular structure remains. A high number of intraepithelial

lymphocytes is observed [30]. This cancer consistently shows microsatellite instability (MSI) with mutations induced by defects in the mismatch repair (MMR) system, which normally corrects errors produced by DNA polymerase during replication. Microsatellite unstable colorectal carcinomas generally have a more indolent clinical course and a more favorable prognosis than microsatellite stable colorectal carcinomas [31].

1.1.2.2. Mucinous adenocarcinoma

This type represents 5 to 15% of colorectal cancers. Its extracellular mucoid material accounts for more than 50% of the tumor mass, and it is also associated with the MSI phenotype [32].

1.1.2.3. Signet-ring cell carcinoma

Very rare, representing 1 to 2.4% of colorectal cancers, this type is defined by the presence of more than 50% of isolated cells containing intracytoplasmic mucin vacuoles that fill the cytoplasm and displace the nucleus toward the cell membrane. It may appear as pools of mucin or as a diffuse infiltrative process with less mucin. In about one-third of cases, this type is associated with the MSI phenotype [33].

1.1.2.4. Adenosquamous carcinoma

For this type to be defined, several small foci of squamous differentiation must be present. These tumors show features of both squamous cell carcinoma and adenocarcinoma, which may appear within the tumor as either separate or intermixed areas [30].

1.1.2.5. Undifferentiated carcinoma

These are rare and genetically distinct tumors, usually associated with the MSI phenotype but lacking morphological, immunophenotypic, or molecular markers of

differentiation. They are associated with a poor prognosis [34].

There are also other types that are very rare and have a poor prognosis, namely the micropapillary and cibriform or poorly diferenciated adenocarcinomas.

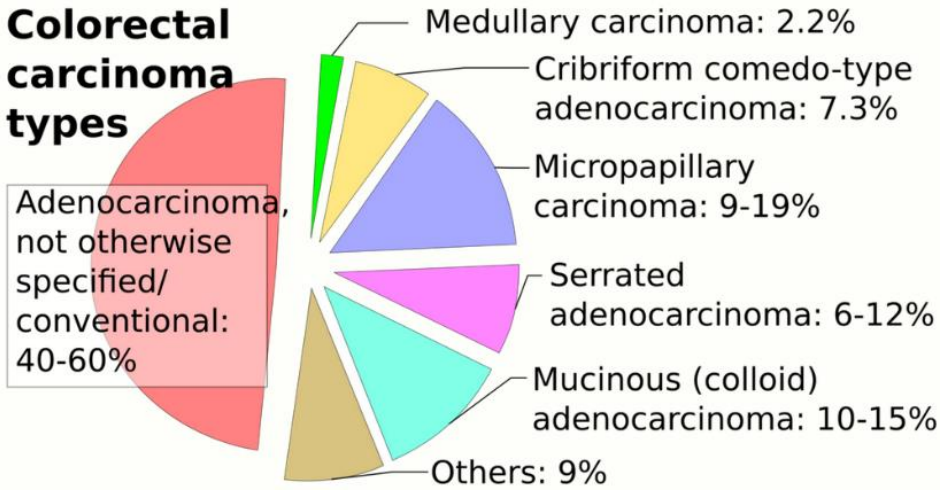

Figure 1.1. Histological Types of colorectal cancer [35]

1.1.3. Risk factors

1.1.3.1. Diet and lifestyle

a. Dietary patterns

Certain diets have been implicated in the onset of colorectal cancer, such as diets high in red meat, high in fat, and low in fiber. The same applies to certain cooking methods, including frying and grilling. Studies have shown that these foods generate fat- and protein-rich compounds known as advanced glycation end products (AGEs)**.** These compounds induce oxidative stress and chronic inflammation, thereby creating a favorable environment for colorectal carcinogenesis [1].

b. Sugar

A high sugar intake can lead to obesity, insulin resistance, and type 2 diabetes. It also promotes the production of AGEs, thereby causing chronic inflammation and increased sensitivity of the colorectal epithelium, ultimately leading to cellular damage [36].

c. Obesity and sedentary lifestyle

These two factors are closely associated with the development of colorectal cancer. A study conducted in 2017 showed that for every 5 kg/m² increase in body mass index (BMI), the risk of developing colorectal cancer increased by 30% in men and by 12% in women [37]. Obesity is a major contributor to metabolic syndrome, elevated IGF-1 levels, and insulin resistance, all of which are associated with a higher risk of CRC [38].

1.1.3.2. Chronic Inflammatory Bowel Diseases (IBD)

IBD results from genetic predispositions, immune disorders, or environmental factors and is represented by two distinct entities:

- **Crohn's disease (CD):** characterized by chronic inflammation that can extend from the mouth to the anus and is transmural in nature.
- **Ulcerative colitis (UC):** a chronic non-transmural inflammation of the rectum that extends upward throughout the colon.

CD and UC share common histopathological features, including the presence of lymphoplasmacytic infiltrates and neutrophils, which are responsible for maintaining inflammation. In some cases, this persistent inflammation leads to colorectal cancer or high-grade dysplasia.

1.1.3.3. Hereditary Factors

Approximately 28% of early-diagnosed colorectal cancers have a family history. Patients with a first-degree relative affected by CRC have a fourfold increased risk of developing the disease [39,40].

The most well-known hereditary syndromes that confer a particularly high risk of CRC are Lynch syndrome and familial adenomatous polyposis (FAP, linked to APC gene mutations) (APC) [1].

1.1.4. Oncogenesis

Colorectal cancer is a heterogeneous disease in which the transformation of a normal epithelial cell into a cancerous cell is linked to the successive accumulation of

mutations in oncogenes and tumor suppressor genes [41].

There are three main mechanisms of colorectal carcinogenesis:

1.1.4.1. Chromosomal Instability (CIN or LOH)

This type of cancer represents about 85% of sporadic cases and is characterized by the accumulation of numerous mutations in oncogenes and tumor suppressor genes, such as the inactivation of the APC (adenomatous polyposis coli) gene, which initiates colorectal carcinogenesis. Other mutations frequently observed include KRAS, P53, PIK3, and TGFβ, all of which promote tumor progression [42].

a. Adenomatous polyposis coli (APC)

The APC gene is part of the Wnt signaling pathway, which regulates cytoplasmic levels of β-catenin. β-catenin, in turn, is involved in maintaining cytoskeletal integrity. A mutation in the APC gene induces elevated levels of β-catenin, leading to weak cell-cell adhesion and reduced migration of cells toward the base of the crypts. As a result, these cells accumulate and form neoplastic polyps in the gastrointestinal tract, thereby increasing the risk of carcinoma.

b. KRAS

KRAS belongs to the MAPK pathway and represents the second most common mutation in CRC. It induces increased Ras activity, accompanied by enhanced ERK activity, which is responsible for the pathogenesis, progression, and cellular proliferation observed in CRC [43].

c. BRAF

BRAF is part of the ERK/MAPK pathway and encodes a serine-threonine kinase of the Raf family. This mutation is also responsible for promoting cell proliferation through

increased kinase activity and is associated with a poor prognosis [44].

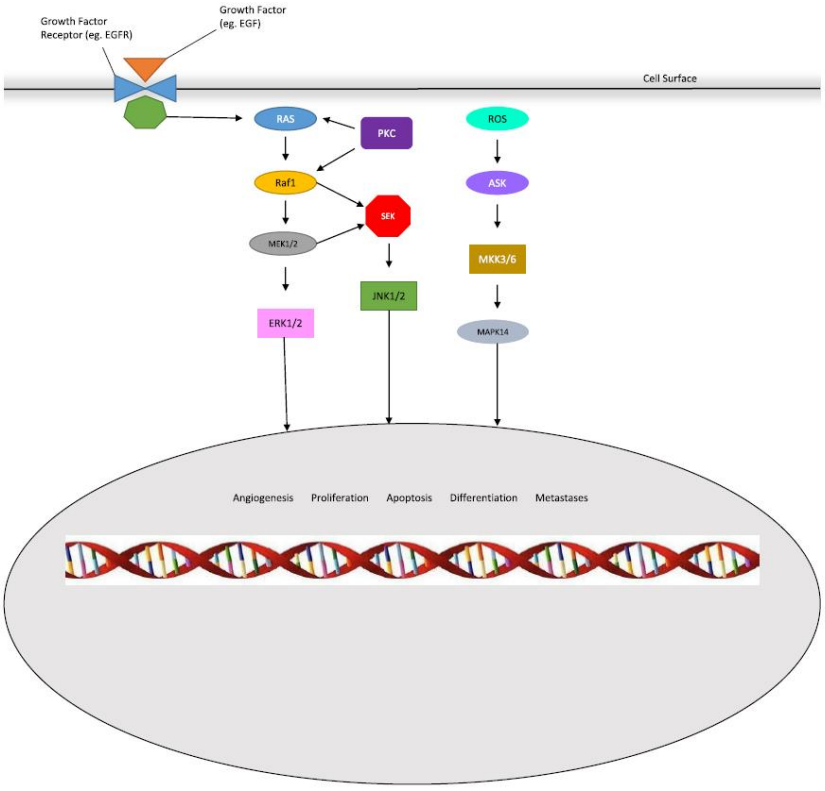

Figure 1.2. MAPK signaling pathways [43]

d. P53

P53 is a protein that maintains cell cycle stability. It halts the cell cycle at the G1/S checkpoint in the presence of mutations and induces apoptosis. However, when P53 itself is mutated, apoptosis is inhibited, thereby favoring CRC pathogenesis [44].

1.1.4.2. Microsatellite Instability (MSI)

Microsatellite instability is a state of genetic hypermutability and instability in highly repeated DNA sequences caused by defects in the DNA mismatch repair (MMR)

system, resulting in a specific tumor phenotype known as dMMR (deficient mismatch repair). This system is composed of several genes responsible for correcting errors in newly synthesized DNA strands, including MLH1, MSH2, MSH6, and PMS2.

MSH2 and MSH6 form a heterodimer called MutSα, which identifies errors on the DNA strand and initiates repair by recruiting another heterodimer, MutLα, formed by MLH1 and PMS2. This heterodimer enables exonuclease activity to remove the erroneous bases, thereby allowing DNA replication to resume with correct base pairing [45].

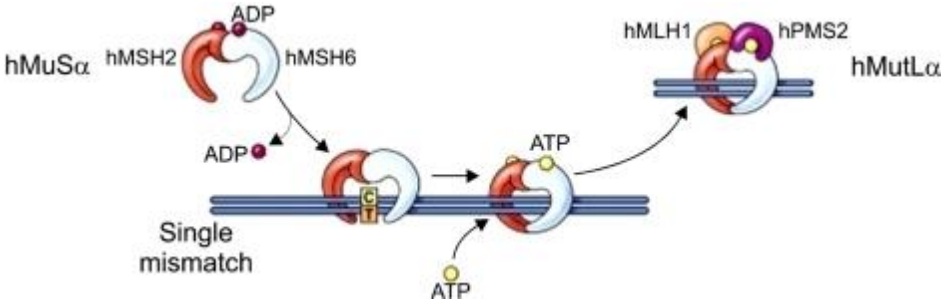

Figure 1.3. Mismatch repair MMR system [45]

Such defects may arise from somatic origins, as in 10–15% of sporadic CRC cases [46] or more rarely from genetic predisposition syndromes such as Lynch syndrome, which accounts for 2–5% of CRC cases [45].

1.1.4.3. CpG Island Methylator Phenotype (CIMP)

CpG islands (CGIs) are short sequences rich in cytosine-guanine dinucleotides, located in the 5’ regions of about half of human genes. Hypermethylation of these CGIs is associated with loss of gene expression and serves as an alternative mechanism for transcriptional inactivation of certain tumor suppressor genes involved in CRC. Nearly half of these genes have been found to be aberrantly methylated in certain sporadic cancers, including Rb, VHL, P16, hMLH1, and BRCA1[47].

Almost all sporadic MSI CRCs are CIMP+ due to hypermethylation of the MLH1 promoter.

1.2. Tumor Microenvironment of Colorectal Cancer

Within the tumor microenvironment, various types of molecular interactions regulate anti-tumor immune responses and strongly modulate inflammation, including:

1.2.1. Cell-cell interactions

This represents one of the most important interactions, occurring between different cell types, and may occur either through direct contact such as:

- Recognition of tumor antigens by T cells via the TCR–MHC interaction.
- Apoptosis mediated by death ligands such as Fas/FasL.
- Activation of NK cells due to loss of MHC or through stress signals such as MICA, which binds to NKG2D and allows NK cells to activate and secrete cytotoxic granules.
- Inhibition of phagocytosis via “do not eat me” signals such as CD47, expressed by tumor cells.
- Transfer of receptors from an immune cell to a tumor cell via trogocytosis, thereby promoting immunosuppression.
- Extravasation of immune cells into the tumor microenvironment.

Cell–cell interactions may also be indirect, as in:

- Antibody-mediated interactions, such as antibody-dependent cellular cytotoxicity (ADCC), where antibodies bound to tumor cells interact with FcγR on NK cells, triggering cytotoxic granule release and tumor cell death.
- Efferocytosis, which is the ability of phagocytes to recognize apoptotic cells and debris displaying phosphatidylserine on their surface. This allows phagocytes to bind via bridging proteins and phagocytose apoptotic material.

1.2.2. **S**oluble immunomodulatory Factors

Cells secrete numerous soluble factors that orchestrate immune responses, including:

- Cytokines (chemokines, interleukins, interferons), the most abundant soluble proteins in the tumor microenvironment. These are secreted by immune cells, tumor cells, or stromal cells, and can exert either anti-tumoral or pro-tumoral effects depending on their nature and target cell.
- Proteases and protease inhibitors, which strongly regulate immune mechanisms by remodeling the microenvironment. Various protease families are present in cell membranes as well as intra- and extracellular spaces.
- Release of receptors in soluble form through proteolytic cleavage, such as soluble FasL (sFasL) released by colon tumor cells, which binds to Fas on T cells and induces their apoptosis.
- Nucleic acids in the tumor microenvironment, which influence anti-tumoral responses.

- Amino acid depletion required for immune responses, e.g., arginine reduction by arginase (highly expressed in myeloid cells) limits nitric oxide (NO) production, thereby impairing a potent anti-tumoral and antimicrobial effector pathway.
- Reactive oxygen species (ROS) and reactive nitrogen species (RNS), present in the tumor microenvironment as a result of metabolic dysregulation and hypoxia, which impact both innate and adaptive immunity.

1.2.3. Tumor Immunomodulation Mediated by the Extracellular Matrix (ECM)

The extracellular matrix regulates cell behavior by controlling adhesion and the spatiotemporal release of soluble factors with key immunomodulatory roles, such as:

- Biomechanical scaffold regulation, controlled by molecules such as fibrillar collagen, which is highly abundant in the colorectal cancer ECM. This makes the ECM extremely dense and rigid, modulating immune cell recruitment and function by blocking cytotoxic T cells while promoting the infiltration of immunosuppressive macrophages.
- Reservoir of proteins, storing secreted soluble factors and regulating their spatial and temporal release near target cells.
- Direct signaling, whereby ECM components are biologically active and directly signal to immune receptors.
- Indirect immunomodulation, mediated by antimicrobial peptides (AMPs) that can exert direct cytotoxic actions on tumor cells.

1.2.4. Tumor Immunomodulation Mediated by the Microbiome

The microbiome is a hallmark of cancer, as it modulates tumor-associated inflammation in several ways:

- Interaction between the microbiota and immune cell populations maintains intestinal immune homeostasis.
- Pro- and anti-tumorigenic microbial inflammation, mediated by specific bacterial strains frequently present in tumors, which may induce persistent inflammation and promote CRC.
- Tumor immunomodulation via microbiome–cell interactions can be induced through several mechanisms, including:
  - Activation of immune cells through PRR stimulation upon detection of bacterial components.
  - PRR activation by bacterial extracellular vesicles (EVs), leading to cytokine secretion.
  - Immunomodulation by short-chain fatty acids (SCFAs) produced during bacterial fermentation.
  - Indirect immunomodulation by bacterial toxins.
  - Induction of cytokine production upon bacterial adhesion to the cell surface [48].

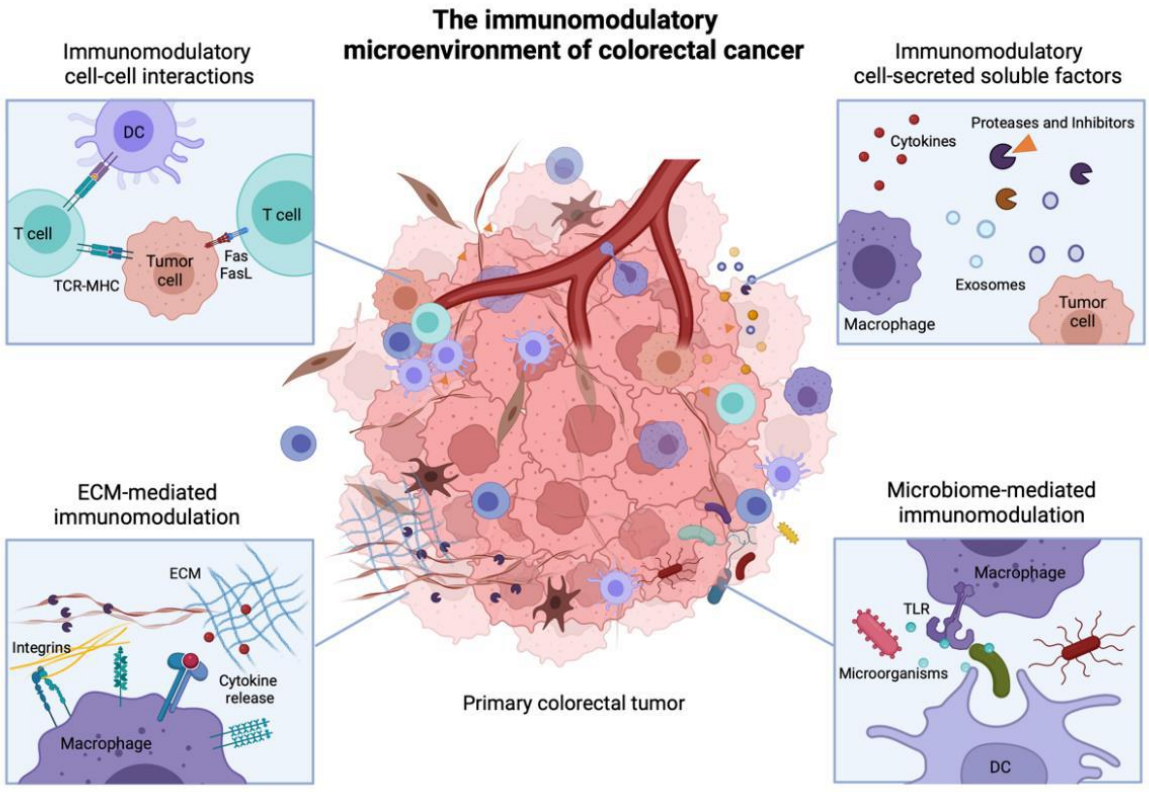

Figure 1.4. Immunomodulatory interactions within the tumor microenvironment in colorectal cancer (CRC) [48]

1.3. Role of the Immune System in Cancer

The immune system is capable of recognizing and eliminating primary tumors. This theory was first proposed in 1909 by Ehrlich [49], Initially rejected due to a lack of evidence, the concept re-emerged as immune surveillance, described by Burnet and Thomas [50] This hypothesis was first studied in immunodeficient mice with spontaneous mutations and later confirmed in humans [51].

The immune system protects the host against tumor formation while also selecting tumors with reduced immunogenicity. This process is referred to as immunoediting, or the "**three Es theory**" (elimination, equilibrium, escape). The elimination phase

corresponds to immune surveillance, during which the immune system identifies and eliminates tumors whose intrinsic tumor suppressor mechanisms have failed [49].

- Elimination phase

This phase consists of eradicating tumor cells that have formed due to defects in intrinsic tumor suppressor mechanisms [49], Both innate and adaptive immune systems are involved. Tumor cells express stress molecules such as calreticulin and tumor antigens on MHC-I, which are presented by DCs to NKT cells and recognized by CD8+ T cells, NK cells, and γδ T cells. Once activated, these cells secrete IFN-γ, which:

i. Recruits additional innate immune cells such as macrophages and granulocytes, which in turn secrete IL-12, TNF-α, IL-1, and ROS with strong anti-tumoral activity.
ii. Exerts anti-tumoral effects by inhibiting proliferation and angiogenesis. Activated CD8+ T cells express co-stimulatory molecules, enabling them to eliminate tumor cells through Fas/FasL and TRAIL pathways, as well as by secreting granzymes and perforins [50].

In cases of partial tumor elimination, a state of equilibrium may develop between the immune system and the progressing tumor [49].

- Equilibrium phase

Tumor cells that survive elimination enter the equilibrium phase, the longest of the three, which can last for years [52], During this phase, tumor cells remain quiescent but continue to accumulate genetic and epigenetic alterations. Equilibrium is maintained by selective pressure from adaptive immune cells, which establish a balance between pro-tumoral cytokines (IL-10, IL-23) and anti-tumoral cytokines (IL-

12, IFN-γ). This process ultimately selects tumor variants capable of resisting, avoiding, or suppressing anti-tumoral immunity, leading to the escape phase [53].

- Escape phase

In this phase, the immune system is no longer able to control the tumor, allowing its progression and the clinical appearance of a detectable mass [49]. Tumor cells lose recognition molecules (MHC-I, NKG2D ligands), becoming less immunogenic, and express resistance molecules such as STAT3, anti-apoptotic proteins (BCL2), and immunosuppressive molecules (TGF-β, IL-10, PD-L1). These factors induce the appearance of CTLA-4 and Tregs, reducing CD8+ T-cell function and inducing apoptosis. Tumor cells also secrete cytokines such as VEGF, IL-6, and M-CSF, which promote angiogenesis [50] by expanding populations of immature myeloid cells. These cells not only help tumors suppress immune responses but also facilitate the construction of new blood vessels for tumor growth and metastasis [54].

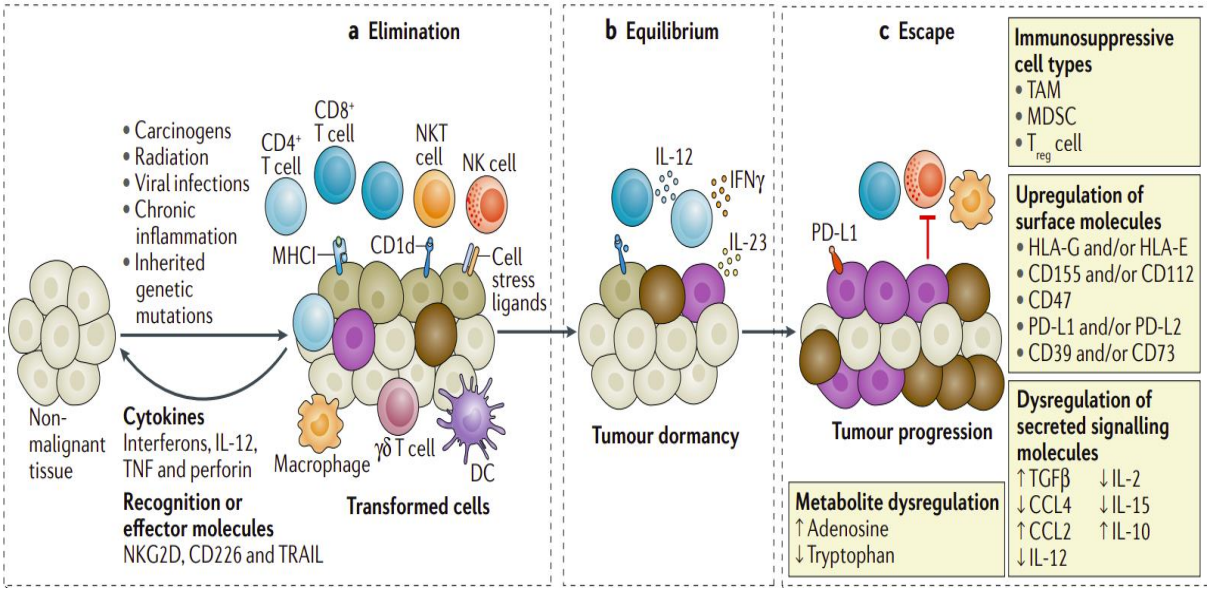

Figure 1.5. Immunoediting mechanism [55]

1.4. Monocytes

1.4.1. General overview

Monocytes are a population of mononuclear leukocytes characterized by their large size, bean-shaped nucleus, and ability to migrate into tissues in response to inflammatory or infectious signals. They play a crucial role in the innate immune system. Monocytes develop in the bone marrow, originating from dividing monoblasts [56]. Once formed, they leave the bone marrow to enter the bloodstream, where they account for 5–10% of leukocytes. They then migrate into peripheral tissues where they differentiate into macrophages or dendritic cells [57], Their primary functions include host defense, phagocytosis and clearance of cellular debris, as well as the production of pro-inflammatory cytokines. Monocytes are identified by the expression of CD14 (a receptor for polysaccharides) and CD16 (FcγRIII) and are subdivided into different subsets [58].

1.4.2. Classification of monocytes

A. Classical monocytes

These monocytes are $CD14^{++}$ $CD16^{-}$ and represent about 90% of the total monocyte population. They express CCR2 and FcγRI (CD64), and produce small amounts of IL-10 following lipopolysaccharide (LPS) stimulation [59], They also exhibit high phagocytic capacity [60] and produce TNF-α, nitric oxide, and reactive oxygen species [61].

B. Intermediate monocytes

This group is $CD14^{++}$ $CD16^{+}$, expresses high levels of CCR5 and CCR2, and low levels of CX3CR1. They rapidly migrate to sites of infection and produce TNF-α, nitric oxide,

and reactive oxygen species [61]. These monocytes are considered pro-inflammatory [58].

C. Non classical monocytes

Also known as patrolling monocytes, these are $CD14^{+}$ $CD16^{++}$, expressing high levels of CX3CR1. They patrol capillaries, eliminate debris, and rarely migrate into tissues [62], Compared to classical monocytes, they display lower phagocytic activity [60].

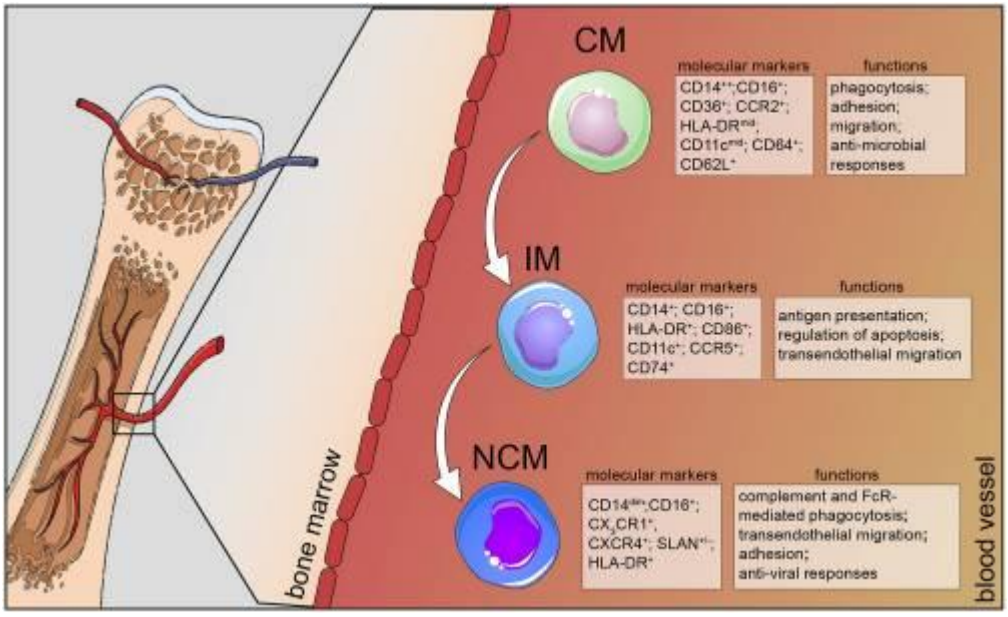

Figure 1.6. Human monocytes subpopulations [10]**.**

1.4.3. Monocyte fate

Monocytes have a short lifespan in the blood, estimated at 2–3 days [63], During inflammation or tissue injury, classical pro-inflammatory monocytes migrate into tissues and differentiate into macrophages. Conversely, non-classical monocytes can reside in non-inflammatory tissues, acquiring dendritic cell-like characteristics [64] [57].

Inflammatory monocytes contribute to immune defense by directly migrating to infected tissues. Pro-inflammatory signals from the infection site induce the migration of monocytes from the bone marrow and circulating classical monocytes via the upregulation of adhesion molecules (integrins, selectins) and chemokines such as CCR2/MCP-1 (monocyte chemoattractant protein-1) on local endothelial cells. This process is known as chemotaxis [58].

Inflammation is associated with the production of cytokines, chemokines, and other mediators, which recruit monocytes. Once recruited, these cells differentiate into macrophages, which can polarize into M1 or M2 phenotypes depending on the Th1/Th2 cytokine balance in the microenvironment [57].

1.4.4. Involvement of Monocytes in the Tumor Microenvironment of Colorectal Cancer

In colorectal cancer (CRC), tumor cells secrete chemokines (e.g., CCL2/MCP-1) that recruit monocytes into the tumor. Once infiltrated, these monocytes predominantly differentiate into tumor-associated macrophages (TAMs)**,** although some retain intermediate phenotypes or evolve into dendritic cells [65]. TAMs derived from monocytes can adopt different functional profiles, ranging from pro-inflammatory M1-like to immunosuppressive M2-like phenotypes. In CRC, the M2 phenotype generally predominates, promoting immune tolerance, tumor cell proliferation, angiogenesis, migration, and metastasis [66] . Functionally, monocytes infiltrating the colorectal TME undergo profound alterations in their phenotype and activity [67]. Exposed to an environment enriched in cytokines (IL-6, TGF-β, IL-10), growth factors (VEGF), hypoxic signals (HIF-1α), metabolic by-products, and ROS [44], their transcriptional

and metabolic profiles are profoundly reprogrammed. A major mechanism of this reprogramming is activation of inflammatory signaling pathways, particularly the NF-κB pathway**,** which regulates transcription of many cytokines, pro-inflammatory enzymes, and surface molecules [68]. Moreover, several studies have shown that tumor-educated monocytes also activate the NLRP3 inflammasome**,** a cytosolic multiprotein platform that responds to danger-associated molecular patterns (DAMPs). This leads to caspase-1 activation and production of mature cytokines such as IL-1β and IL-18**,** which in turn sustain chronic inflammation, a recognized driver of colorectal tumorigenesis [69]. In parallel with these functional changes, monocytes in contact with the TME undergo deep metabolic alterations**.** Tumor signals may drive the accumulation of lipid droplets (LDs)**,** which are not merely inert energy stores but actively modulate inflammatory responses [70]. Indeed, LDs are now recognized as pro-inflammatory signaling platforms**,** involved in the sequestration or production of bioactive lipid mediators [71]. In CRC, the changes given in lipid metabolism of monocytes are often correlated with alterations in phagocytic activity and cytotoxic potential, thereby contributing to tumor progression [72].

1.5. Cholesterol, Triglycerides, and Lipid Droplets: Lipid Metabolism and Immune Implications

Cellular lipid metabolism encompasses the full set of biochemical processes involved in the synthesis, degradation, storage, and transport of lipids within cells [73]. Among these, cholesterol and triglycerides play key roles in membrane structure, cell signaling, and energy supply [74]. Lipid droplets (LDs)**,** dynamic intracellular structures, ensure the storage of these neutral lipids and actively participate in regulating lipid

homeostasis [75]. In the immune system, lipids are essential regulators of immune cell function, particularly monocytes and macrophages, by influencing their activation, polarization, and responsiveness to inflammatory cues. However, in pathological contexts such as cancer, lipid metabolism is profoundly altered. Both tumor cells and tumor-infiltrating immune cells undergo metabolic reprogramming that favors the accumulation of cholesterol and triglycerides, often stored as lipid droplets.

These perturbations contribute to the establishment of an immunosuppressive tumor microenvironment**,** conducive to tumor progression and immune escape [76].

1.5.1. Cholesterol

1.5.1.1. Définition

Cholesterol is an essential lipid for cell survival, predominantly localized in the plasma membrane, where it interacts with other lipids to regulate membrane rigidity, fluidity, and permeability. Beyond its structural role, cholesterol is involved in cell signaling, modulation of membrane trafficking, and host-pathogen interactions, and it also serves as the common precursor of all steroid hormones. Given such crucial functions across diverse physiological contexts, disturbances in cholesterol metabolism are associated with numerous diseases, including cancer [77].

1.5.1.2. Biosynthesis, Transport, and Regulation of Cholesterol

Cholesterol can be synthesized de novo from acetyl-CoA through a cascade of about 30 enzymatic reactions. The rate-limiting steps of this pathway are catalyzed by two major enzymes: 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) and

squalene monooxygenase (SM)**.** In addition to this endogenous synthesis, cells can acquire circulating cholesterol carried by low-density lipoproteins (LDL), via the LDL receptor (LDLR) localized on the basal membrane of polarized cells such as enterocytes (intestine) and hepatocytes (liver).

Dietary or biliary cholesterol can also be absorbed by these cells through the transporter NPC1L1 (Niemann–Pick C1-like 1)**,** assisted by flotillins on the apical membrane (Figure 1.7) [77].

To maintain cellular homeostasis, cholesterol metabolism is tightly regulated, mainly through two mechanisms:

- **Transcriptional regulation**: Sterol Regulatory Element-Binding Proteins (SREBPs), especially SREBP-2**,** play a central role. Under cholesterol depletion, SREBP-2 is activated and stimulates transcription of genes encoding HMGCR, LDLR, and other enzymes involved in cholesterol biosynthesis.
- **Feedback control by intracellular cholesterol**: When cholesterol levels increase, SREBP maturation is inhibited, reducing expression of genes involved in synthesis and uptake. Furthermore, free cholesterol can stimulate ACAT (acyl-CoA:cholesterol acyltransferase, also known as SOAT) activity, promoting its esterification and storage in lipid droplets within the cytoplasm or incorporation into lipoproteins for secretion [78].

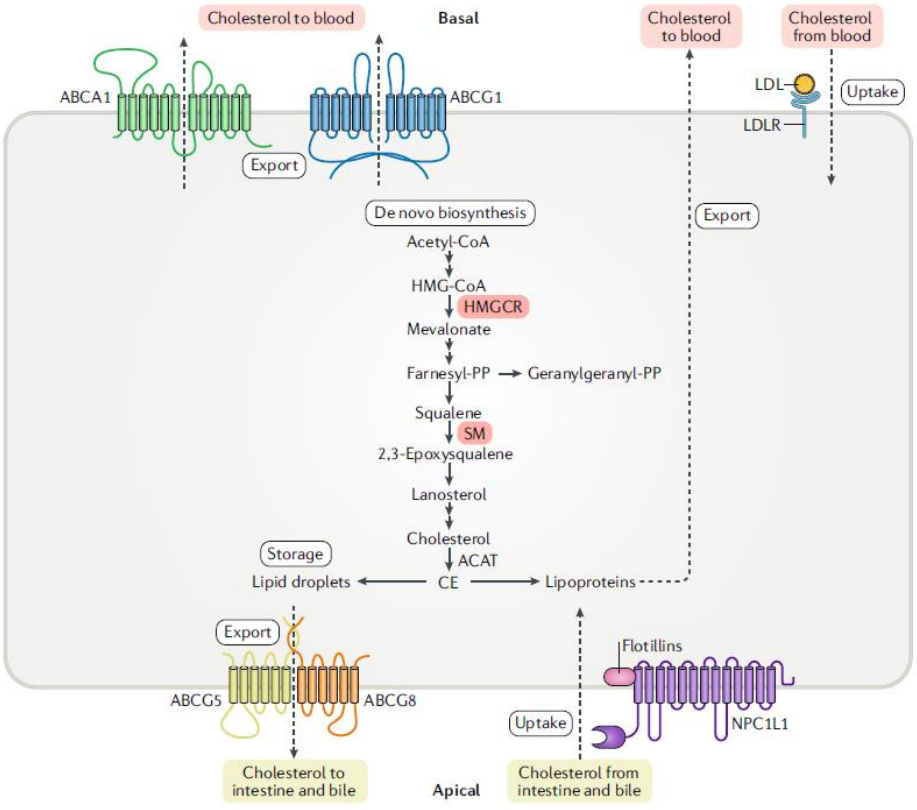

Figure 1.7. Biosynthesis and transport of cholesterol [77]

1.5.1.3. Cholesterol Metabolism in Macrophages

Macrophages rapidly and profoundly reprogram their cholesterol metabolism in response to activation signals, particularly those derived from pathogen recognition receptors (PRRs) and other pro-inflammatory stimuli, in order to support host defense mechanisms. This reprogramming depends on the nature of the signal received and leads to distinct metabolic profiles depending on the context. Studies have shown that activation via MyD88-dependent PRRs induces a rapid increase in cholesterol biosynthesis, leading to overall accumulation of cellular cholesterol[79]. However,

excessive accumulation of free cholesterol can cause severe cellular dysfunction and activate the inflammasome, resulting in interleukin-1β (IL-1β)-mediated inflammation [78].

1.5.2. Lipid droplets (LDs)

1.5.2.1. Definition and composition

Lipid droplets are dynamic cytoplasmic organelles, nearly ubiquitous across cell types. They are characterized by their unique composition: a hydrophobic core of neutral lipids, mainly sterol esters (SE) and triacylglycerols (TAG), surrounded by a phospholipid monolayer separating them from the cytosol (Figure 1.8) [75].

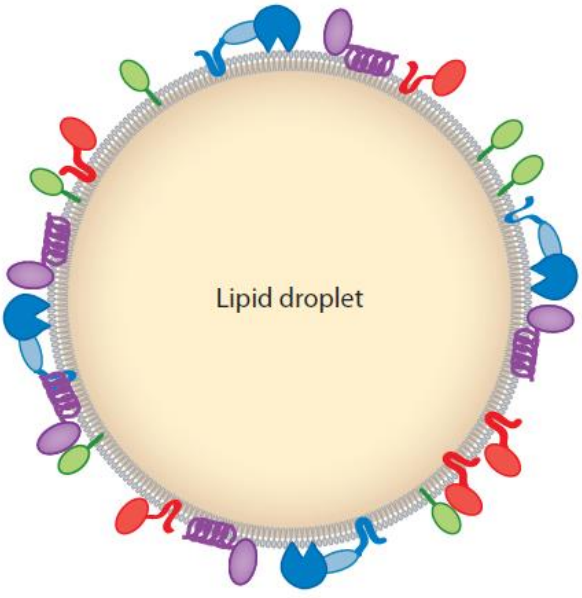

Figure 1.8. Structure of lipid droplets [75]

1.5.2.2. Origin of stored lipids : focus on triglycerides

- Triglycerides

Also known as triacylglycerols, these are the main storage forms of fatty acids in cells. They consist of a glycerol backbone esterified to three fatty acids and are primarily stored as lipid droplets [80]. TAGs are mainly synthesized in the endoplasmic reticulum, catalyzed by the enzymes acyl-CoA: diacylglycerol acyltransferase (DGAT)**,** specifically DGAT1 and DGAT2 [75] .

1.5.2.3. Lipid droplets functions

Lipid droplets are involved in many cellular functions, including storage of lipids for energy production and membrane synthesis, viral replication, and degradation of certain proteins [75]. Increasingly, they are recognized as regulators directly or indirectly of other cellular processes, ranging from storage of hydrophobic vitamins and signaling precursors to managing endoplasmic reticulum (ER) stress, oxidative stress, and production of inflammatory mediators [80].

1.5.2.4. Lipid Droplet Accumulation During Inflammation and Cancer

In chronic pathological states such as persistent inflammation, infections, and particularly in the tumor microenvironment (TME)**,** a marked accumulation of lipid droplets is often observed in myeloid cells, notably monocytes and macrophages. This accumulation results from a complex metabolic reprogramming involving increased lipid uptake, stimulation of neutral lipid synthesis (triacylglycerols and cholesterol esters), and alterations in lipid storage and mobilization pathways.

TME signals—such as pro-inflammatory cytokines, growth factors, hypoxia, excess tumor-derived lipids, and lactate—modulate the expression and activity of several metabolic regulators: fatty acid transporters **(**CD36, FATP**),** triglyceride synthesis enzymes **(**DGAT1/2**),** and cholesterol esterification enzymes **(**ACAT/SOAT**).** At the same time, transcriptional pathways such as SREBPs (for neutral lipids) and LXR receptors can be reprogrammed, leading either to increased lipid biosynthesis or altered lipid distribution (release vs. storage). The outcome is enhanced biogenesis of lipid droplets from the endoplasmic reticulum.

In the TME, a high lipid droplet burden can contribute to two seemingly contradictory but complementary effects:

- **Metabolic support** for immune cells in nutrient-poor environments (promoting survival and specific functions).
- **Functional reprogramming** toward an immunosuppressive phenotype (reduced phagocytosis, impaired antigen presentation), particularly in TAMs [74].

During inflammation, lipid droplet accumulation regulates inflammatory responses through their interaction with key signaling pathways, notably NF-κB and the NLRP3 inflammasome**.**

- NF-κB activation, often triggered by PRR signals (e.g., TLR4/MyD88), acts as a “priming” step by inducing transcription of pro-inflammatory genes, including NLRP3 and pro-IL-1β. In parallel, metabolic changes associated with this activation promote lipid droplet formation, particularly via increased triacylglycerol synthesis and cholesterol esterification.

Excessive lipid load, oxidized lipid derivatives, and cholesterol crystals can provide the necessary “triggering” signals (signal 2) for NLRP3 inflammasome activation**.** This occurs through mechanisms such as enhanced reactive oxygen species (ROS) production, membrane perturbation, or lysosomal stress. Collectively, these events lead to caspase-1 activation and maturation of pro-inflammatory cytokines like IL-1β [81].

1.6. Inflammasome

1.6.1. Definition and components

The NLRP3 inflammasome is a key element of innate immunity. It consists of the NLRP3 protein (NLR family pyrin domain-containing 3)**,** the adaptor ASC (apoptosis-associated speck-like protein), which contains a caspase recruitment domain (CARD) and pro-caspase-1**,** the effector component.

The NLRP3 protein has three functional domains:

- an N-terminal pyrin domain (PYD)**,** which interacts with the PYD of ASC to initiate inflammasome assembly,
- a central nucleotide-binding and oligomerization domain (NACHT)**,** essential for activation, and a C-terminal leucine-rich repeat (LRR) domain, recently shown to be non-essential for NLRP3 inflammasome assembly.

The adaptor ASC recruits and binds pro-caspase-1 via their respective CARD domains, triggering its auto cleavage and activation. Activated caspase-1 then

promotes the release of the inflammatory cytokines interleukin-1β (IL-1β) and interleukin-18 (IL-18)**,** and cleaves gasdermin D (GSDMD)**,** thereby inducing a specific form of programmed cell death called pyroptosis [68].

1.6.2. Mecanisms of activation

1.6.2.1. Priming

The priming phase of NLRP3 inflammasome activation is mainly orchestrated by the transcription factor NF-κB**,** particularly its p65/RelA subunit. When a pattern recognition receptor (PRR) such as TLR4 is stimulated by lipopolysaccharide (LPS), the adaptor pathway MyD88 activates the IKK complex, which phosphorylates the inhibitor IκBα. Its degradation releases the p65/RelA–p50 dimer, allowing it to translocate into the nucleus [82].

Once in the nucleus, p65/RelA binds to κB sequences in the promoters of genes encoding NLRP3, pro-IL-1β, and pro-IL-18, thereby inducing their transcription and preparing the cell for rapid activation upon receiving the second signal. Phosphorylation of p65, particularly at serine 536, enhances its ability to recruit transcriptional co-activators and sustain expression of these pro-inflammatory genes [83].

1.6.2.2. Activation

After the priming phase, which ensures NLRP3 and pro-cytokine expression, a second signal is required to trigger full inflammasome assembly. This signal is provided by

various danger stimuli pathogens, stress signals, or metabolic disturbances that alter cellular homeostasis and are indirectly sensed by NLRP3.

Key mechanisms include:

- intracellular Ca^{2+} flux,
- K^+ efflux,
- increased ROS production,
- lysosomal rupture, release of endogenous damage-associated molecular patterns (DAMPs) or pathogen associated molecular patterns (PAMPs), and mitochondrial dysfunction.

Well-known stimuli include nigericin, extracellular ATP, and cholesterol crystals, all capable of inducing ionic disturbances or cellular stress that activate NLRP3 [68,84]. Lipid droplet accumulation, rich in triglycerides and cholesterol esters, is also known to activate the inflammasome, often associated with:

increased mitochondrial ROS due to lipid overload, the presence of cholesterol crystals that disrupt lysosomes, and metabolic reprogramming that modifies cell signaling and facilitates activation of pro-inflammatory pathways such as NF-κB and NLRP3 [85].

Once assembled, the inflammasome induces proteolytic cleavage of inactive pro-caspase-1 into active caspase-1. Caspase-1 then processes pro-IL-1β and pro-IL-18 into their mature, biologically active forms. Mature IL-1β is a major pro-inflammatory mediator, critical for recruiting innate immune cells to the infection site and modulating

adaptive responses. Mature IL-18 stimulates interferon-γ (IFN-γ) production and enhances cytolytic activity of NK cells and T lymphocytes.

In parallel, caspase-1 activation cleaves Gasdermin D (GSDMD), releasing its N-terminal domain, which inserts into the plasma membrane to form pores. These pores cause pyroptosis a pro-inflammatory form of cell death characterized by cell swelling, membrane rupture, and massive release of inflammatory mediators. While this process amplifies immune responses, excessive activation can cause tissue damage [84].

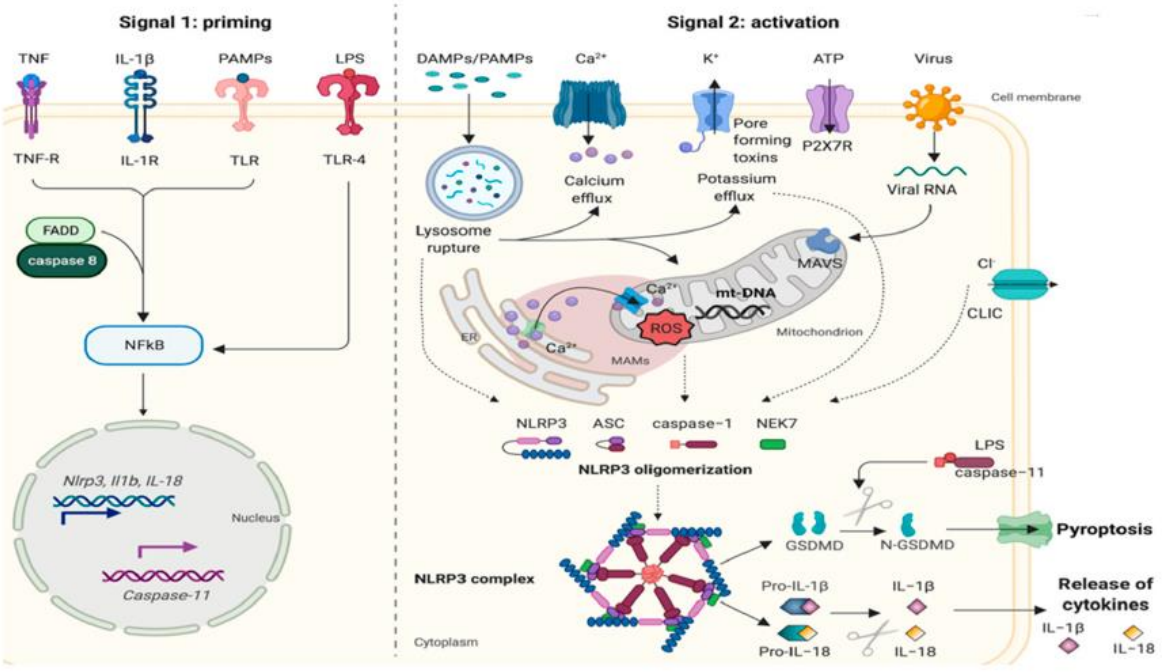

Figure 1.9. priming and activation of NLRP3 inflammasome [68]

1.6.3. Inflammasome and colorectal cancer

The inflammasome, particularly the NLRP3 complex, plays a central role in intestinal inflammation and the development of colorectal cancer (CRC). Its activation leads to the production of pro-inflammatory cytokines (IL-1β, IL-18) and promotes various immune responses [68].

- **Pro-tumoral mechanisms:** Chronic activation of NLRP3 stimulates epithelial cell proliferation and survival, angiogenesis, and tumor invasion. High expression of NLRP3 in human CRC tissues is associated with invasion, migration, and poor prognosis. Polymorphisms in the NLRP3 gene are linked to increased IL-1β/IL-6 levels and reduced patient survival [86].

- **Anti-tumoral mechanisms and protective role:** Conversely, several murine studies have shown that the absence of Nlrp3, Asc, Caspase-1/11, or IL-18 makes mice more susceptible to CRC [87]. NLRP3-derived IL-18 enhances the cytotoxic activity of hepatic NK cells and limits metastatic growth. A hyperactive NLRP3 can even confer resistance to colitis and CRC [88].

1.7. Metformin

1.7.1. Definition

Metformin (METF hydrochloride, 1,1-dimethylbiguanide) is a synthetic insulin-sensitizing semi-biguanide, consisting of two methyl groups bound to a nitrogen nucleus, as shown in (Figure 1.10). It is a hypoglycemic agent originally derived from the plant *Galega officinalis*, known for its potent normoglycemic properties [89] , Also referred to as Glucophage, it is prescribed to approximately 100 million type 2 diabetes (T2D) patients annually [90].

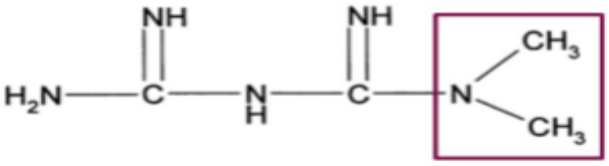

Figure 1.10. chemical structure of metformin [91]

1.7.2. Mechanism of Action of Metformin in Diabetes

Metformin improves insulin sensitivity in the liver, muscles, and adipose tissue.

- In the liver, it inhibits hepatic glucose production and reduces plasma glucose by 25–30% through decreased gluconeogenesis.
- It activates the insulin receptor substrate 2 **(**IRS2) signaling pathway, increases glucose uptake by enhancing GLUT-1 (glucose transporter-1) translocation, and acts at the mitochondrial level by inhibiting the respiratory chain, leading to

decreased ATP (adenosine triphosphate) and activation of AMPK (5' AMP-activated protein kinase), thereby suppressing gluconeogenesis [92,93].

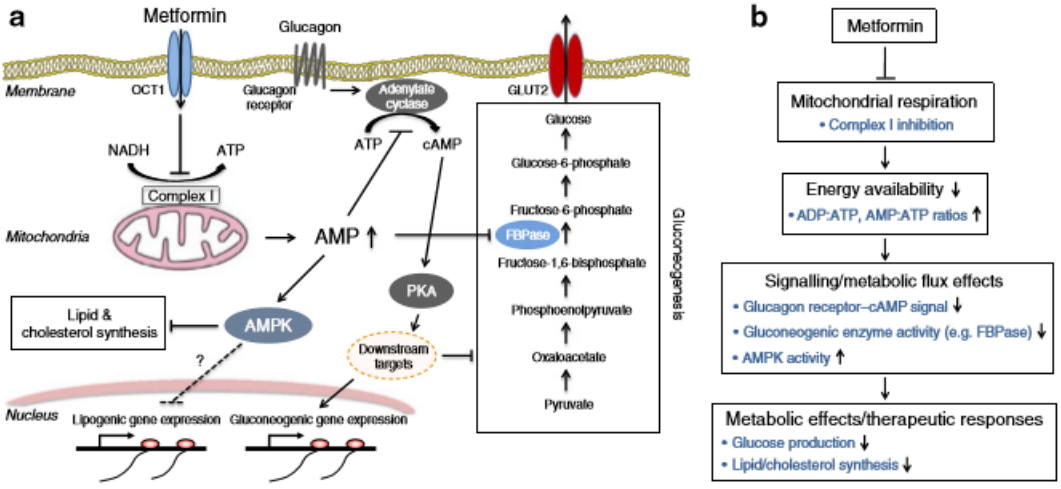

Figure 1.11. The action of metformin on hepatic cells [93]

1.7.3. Link between Type 2 Diabetes and Colorectal Cancer

Type 2 diabetes (T2D) is a metabolic disorder characterized by chronic hyperglycemia, insulin resistance, and compensatory hyperinsulinemia [94], These metabolic alterations contribute to the development and progression of several cancers, including CRC. Major mechanisms include:

1.7.3.1. **Hyperinsulinemia and IGF-1**: Excess insulin activates the insulin receptor (IR) and stimulates production of insulin-like growth factors (IGF-1), which promote cell proliferation and inhibit apoptosis through PI3K/AKT and MAPK pathways [95].

1.7.3.2. **Hyperglycemia**: Provides a favorable energy environment for tumor cells by fueling aerobic glycolysis **(**Warburg effect**).** It also increases ROS production, both directly via glucose auto-oxidation and indirectly through advanced glycation end-

products (AGEs) that activate RAGE receptors [73]. These processes cause DNA damage and activate pro-inflammatory factors such as NF-κB, thereby promoting carcinogenesis [96].

1.7.3.3. **Insulin Resistance**: Disrupts energy metabolism and results in excess circulating growth factors, creating a pro-inflammatory and proliferative tumor microenvironment [97].

1.7.4. Mechanism of Action of Metformin in Colorectal Cancer

Studies have shown that, among type 2 diabetic patients, metformin treatment is associated with an approximate 37% reduction in CRC risk [98]. The anti-tumoral effect of metformin is explained by both its direct effect on cancer cells through inhibition of multiple pathways involved in metastasis and survival and its indirect effect of maintaining physiological glucose and insulin levels [89].

1.7.4.1. Molecular mecanisms

1.7.4.1.1. **AMPK activation**: Metformin activates AMPK, which regulates energy metabolism, inhibits the mTOR pathway, and decreases protein and lipid synthesis necessary for tumor growth [99].

1.7.4.1.2. **Metformin and mTOR:** The mTOR protein regulates cellular energy homeostasis by modulating protein synthesis and autophagy. Activated AMPK inhibits mTOR activity through phosphorylation of the tumor suppressor complex TSC2, leading to AMPK-dependent inhibition of mTOR signaling. In addition, mTOR can also be inhibited independently of AMPK via metformin-mediated suppression of IGF-1 signaling [100].

1.7.4.1.3. **Effect on cancer stem cells (CSCs):** Metformin reduces CSC markers (e.g., CD44, CD133, Lgr5) in CRC, thereby impairing their sphere- and tumor-forming capacity [101].

1.7.4.1.4. **Anti-inflammatory effect:** Metformin inhibits pathways such as NF-κB, TLR4/MyD88, and MAPK**,** thereby reducing production of pro-inflammatory cytokines and reshaping the immune microenvironment [102].

1.7.4.1.5. **Effect on lipid/cholesterol metabolism:** Certain studies show that metformin reduces HMG-CoA reductase expression and influences the mevalonate pathway, thereby limiting cholesterol and its derivatives necessary for tumor growth [101].

- **Antiproliferative and pro-apoptotic effects**: AMPK activation strongly suppresses cell proliferation in both malignant and non-malignant cells. Several mechanisms are involved:
- **Cell cycle regulation**: Upregulation of the p53–p21 **axis** and reduction of Cyclin D1 levels, leading to inhibition of cyclin-dependent kinases (CDKs), thereby inducing G1 cell cycle arrest.
- **Protein synthesis inhibition**: Mediated primarily via suppression of the mTOR pathway as previously described [99].

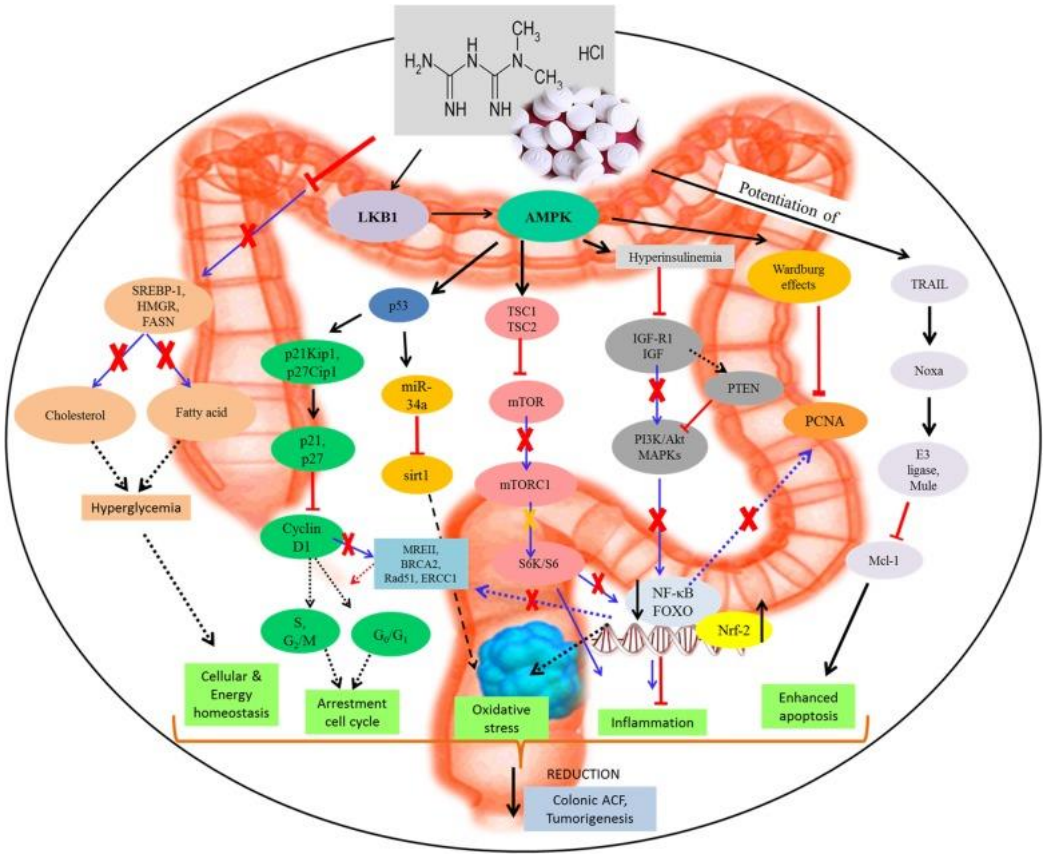

Figure 1.12. Metformin effect on colorectal cancer [99]

1.8. Problematic and objectives

1.8.1. Problematic

Colorectal cancer (CRC) remains one of the most frequently diagnosed malignancies and a leading cause of cancer-related mortality worldwide. Beyond the genetic alterations occurring within tumor cells, it is now well established that tumor progression is strongly influenced by the tumor microenvironment (TME), a highly complex ecosystem composed of immune cells, inflammatory mediators, metabolic factors, and soluble signals released by cancer cells.

Among the immune cell populations present in the TME, monocytes and macrophages play a pivotal role due to their remarkable functional plasticity and their ability to rapidly adapt to environmental cues. In the context of CRC, tumor-derived soluble factors, commonly studied through tumor-conditioned medium (TCM), profoundly reshape monocyte phenotype, metabolism, and effector functions. This immunometabolic reprogramming may contribute to chronic inflammation, impairment of antitumor immune responses, and the establishment of a microenvironment that favors tumor growth and progression.

Increasing evidence highlights the central role of chronic inflammation and metabolic dysregulation—particularly alterations in lipid metabolism and lipid droplet accumulation in the activation of key pro-inflammatory pathways such as nuclear factor kappa B (NF-κB) and the NLRP3 inflammasome. Activation of the NF-κB/NLRP3/interleukin-1β (IL-1β) axis represents a critical mechanistic link between

innate immunity, inflammation, and colorectal tumorigenesis, especially within myeloid cells exposed to tumor-derived signals.

In parallel, metformin, a widely prescribed antidiabetic drug, has attracted growing interest beyond its metabolic effects. Emerging data suggest that metformin may exert anti-inflammatory and immunomodulatory actions by influencing NF-κB signaling, inflammasome activation, and cellular lipid metabolism. However, the precise mechanisms by which metformin modulates monocyte responses within the colorectal tumor microenvironment particularly in relation to lipid droplet formation and NLRP3 inflammasome activation remain incompletely understood.

Therefore, elucidating the interactions between colorectal cancer–derived TCM, monocyte lipid metabolism, activation of the NF-κB/NLRP3/IL-1β axis, and the potential modulatory role of metformin is essential for a deeper understanding of tumor–immune crosstalk and for identifying novel immunometabolic strategies in colorectal cancer.

1.8.2. Objectives

In this study, we aimed to:

1. Investigate the effect of colorectal tumor-conditioned medium (TCM) on monocytes, focusing on their functional and phenotypic alterations.
2. Analyze the impact of metformin on monocytes exposed to TCM, particularly regarding their lipid and cholesterol metabolism.
3. Assess the formation and accumulation of lipid droplets in monocytes under TCM stimulation and metformin treatment.
4. Explore the activation of the NF-κB pathway and its implications in the regulation of monocyte inflammatory responses.
5. Evaluate the activation of the NLRP3 inflammasome and the secretion of pro-inflammatory cytokines, especially IL-1β, in the tumor context.
6. Characterize the phenotypic subsets of monocytes (classical, intermediate, and non-classical) in response to TCM and metformin treatment.

1.8.3. Aim

The aim of this work is to elucidate how the colorectal tumor microenvironment, through its conditioned medium, shapes monocyte phenotype, inflammatory signaling, and lipid metabolism, and to determine how metformin may modulate these processes with a particular focus on NF-κB, NLRP3, IL-1β, and lipid droplet dynamics.

CHAPTER 2: Metformin inhibits NF-κB p65/RelA–NLRP3 inflammasome–IL-1β axis, attenuates lipid droplet accumulation, and reprograms CD14/CD16 expression in monocytes exposed to colorectal tumor-conditioned medium

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Metformin inhibits NF-κB p65/RelA–NLRP3 inflammasome–IL-1β axis, attenuates lipid droplet accumulation, and reprograms CD14/CD16 expression in monocytes exposed to colorectal tumor-conditioned medium

Nihel Chahinez Djebri [a], Souad Zoudji [a], Aida Messaoud [a], Rabia Messali [a], Salim Loudjedi [a,b], Mourad Aribi [a,*]

[a] Laboratory of Applied Molecular Biology and Immunology, W0414100, University of Tlemcen, 13000 Tlemcen, Algeria
[b] Surgery Department B of Tlemcen University Hospital Center, 13000 Tlemcen, Algeria

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ABSTRACT

Background: Colorectal cancer (CRC) generates a complex tumor microenvironment (TME) known to profoundly alter the function and phenotype of immune cells, including monocytes. Key aspects of this environment can be mimicked by tumor-conditioned medium (TCM). Metformin has emerged as a promising candidate to counteract TCM-induced immune and inflammatory dysregulation. Therefore, this study aimed to evaluate the effects of metformin on NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome expression, monocyte subset distribution, and lipid droplet (LD) accumulation upon exposure to colorectal TCM.

Methods: Assays were performed on primary human monocytes exposed to colorectal TCM in the presence or absence of metformin.

Results: TCM significantly increased nitric oxide (NO, $p < 0.001$) and hydrogen peroxide (H_2O_2, $p < 0.001$) production, intracellular free calcium ions ($_{if}Ca^{2+}$) ($p < 0.001$) levels, expression of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB p65/RelA, $p < 0.001$), NLRP3 ($p < 0.01$), and interleukin-1 beta (IL-1β, $p < 0.0001$), as well as intracellular triacylglycerols ($_{i}$TAGs, $p < 0.05$), total cellular cholesterol content ($_{tcc}$CHOL, $p < 0.001$), and lipid droplet accumulation ($p < 0.05$). It also impaired phagocytic activity ($p < 0.05$) and altered monocyte phenotype, along with a shift toward a $CD14^{low}$, $CD16^{high}$ phenotype ($p < 0.0001$ for both markers). Notably, metformin treatment exerted broad and significant reversing effects, specifically on respiratory burst (NO, $p < 0.05$; H_2O_2, $p < 0.01$), $_{if}Ca^{2+}$ levels ($p < 0.01$), NF-κB p65/RelA and NLRP3 expression, and IL-1β production ($p < 0.0001$ for all), and the lipid droplet accumulation ($p < 0.0001$), $_{i}$TAGs ($p < 0.001$), and $_{tcc}$CHOL ($p < 0.01$). Metformin also significantly restored CD14 expression ($p < 0.05$) and increased $CD14^{+}$ monocyte frequency ($p < 0.01$), while reducing CD16 expression ($p < 0.05$) and $CD16^{+}$ monocyte frequency ($p < 0.01$). However, it had no significant effect on phagocytosis in TCM-exposed monocytes ($p > 0.05$).

Conclusions: Our findings highlight metformin's selective immunometabolic reprogramming capacity in monocytes exposed to colorectal tumor-derived signals, supporting its potential as a context-specific immunomodulator. This study lays the groundwork for future translational research on metformin as an adjunctive agent in inflammation-driven tumor settings.

1. Introduction

Colorectal cancer ranks among the most prevalent and deadly cancers worldwide [1]. In addition to genetic and epigenetic alterations, its progression is strongly shaped by the tumor microenvironment [2], a complex network of cytokines, chemokines, metabolites, and other soluble mediators released by tumor and stromal cells [3,4]. These factors not only support tumor growth and immune evasion but also exert profound effects on the function and phenotype of surrounding immune cells [5]. Among its key effector cells, monocytes play a central role in pathogen sensing, phagocytosis, and the orchestration of inflammatory responses [6–9]. Their remarkable plasticity allows them to

* Corresponding authors at:Laboratory of Applied Molecular Biology and Immunology, W0414100, Imama-Mansourah, Rocade # 2, and Department of Biology, Abou-Bekr Belkaïd University of Tlemcen, PO Box: 262, Imama-Mansourah, Tlemcen 13000, Algeria.
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dynamically adapt to environmental cues, particularly within the tumor context. The functional distribution of monocyte subsets, often characterized by CD14 and CD16 expression [10], reflects their activation state and determines whether they adopt proinflammatory or tissue-reparative functions [11].

While the tumor microenvironment is highly complex and shaped by diverse cellular components, it can be partially mimicked by tumor-conditioned media [12], which contains a mixture of cytokines, metabolites, and danger-associated molecular patterns (DAMPs), capable of influencing the behavior and polarization of immune cells, including monocytes. These factors can reprogram monocyte function, notably by modulating intracellular inflammatory mediators such as the NLRP3 inflammasome and the transcription factor NF-κB [13,14]. Of note, certain transformed cancer cells have been reported to influence the metabolic landscape of immune cells, potentially affecting lipid metabolism and storage [15]. However, the extent to which tumor-derived soluble factors modulate lipid droplet accumulation in monocytes re- mains to be fully elucidated.

Given the potential of the tumor microenvironment to reprogram immune cell metabolism and function, there is growing interest in pharmacological strategies capable of counteracting these effects are of growing interest. Among them, metabolic modulators such as metformin (1,1-dimethylbiguanide hydrochloride), widely used as a first-line treatment for type 2 diabetes, have garnered attention for their broad actions. Beyond its glucose-lowering effect, metformin has been shown to exert immunomodulatory effects in diverse experimental settings, including cancer [16]. Moreover, it has been reported to influence lipid metabolism and mitigate inflammatory responses [17], positioning it as a promising candidate for modulating immune cell dysfunctions observed in the colorectal cancer microenvironment [18].

In particular, metformin has been shown to interfere with key inflammatory signaling pathways such as NF-κB and the NLRP3 inflammasome, both of which play central roles in chronic inflammation and cancer-related immune dysregulation [19,20]. NF-κB, a nuclear transcription factor, governs the expression of various proinflammatory genes, while NLRP3, a component of the inflammasome complex, promotes caspase-1 activation and thereby the maturation and release of interleukin-1β (IL-1β) [21], a key effector cytokine in inflammation and in the immune dysfunction associated with tumors [22,23]. Importantly, recent findings suggest that metformin may suppress the activation of NF-κB through the inhibition of reactive oxygen species (ROS) and the activation of AMP-activated protein kinase (AMPK), thereby indirectly dampening NLRP3 inflammasome signaling [18,24]. However, the direct link between metformin and NLRP3 inflammasome regulation, particularly in primary human monocytes exposed to tumor-derived conditions, remains insufficiently understood and largely unexplored. Addressing this critical gap is the central aim of our study. Building on this context, the present study investigates the effects of metformin on NLRP3 inflammasome expression, monocyte subset distribution, and lipid droplet accumulation in response to colorectal tumor-conditioned medium.

2. Materials and methods

2.1. Experimental design and ethical compliance

All procedures involving human-derived materials and *in vitro* experimentation were conducted in strict accordance with institutional ethical guidelines. The study received prior approval from the local Ethical Committee of Tlemcen University, and informed written consent was obtained prior to sample collection. Experiments were carried out on four groups of monocytes: (i) metformin-untreated and tumor-conditioned medium (TCM)-unexposed, (ii) metformin-treated and TCM-unexposed, (iii) metformin-untreated and TCM-exposed, (iv) metformin-treated and TCM-exposed. Each experiment was independently repeated at least four times and conducted in duplicate or triplicate. The study flowchart and design are summarized in Fig. 1.

2.2. Preparation of TCM

TCM was prepared from primary colorectal cancer cells isolated from site-specific tumor specimens obtained from patients undergoing surgery at the Surgery Department B of CHU Tlemcen. Tissue samples were processed immediately after surgical resection to ensure cell viability and integrity. The specimens underwent enzymatic digestion using 0.25 % trypsin at 37 °C for 18 h, followed by differential centrifugation to isolate viable tumor cells [25,26]. The isolated cells were then seeded at a density of 2×10^5 cells/mL in T75 culture flasks containing RPMI 1640 medium supplemented with 10 % fetal bovine serum (FBS) and antibiotics, including gentamycin at a final concentration of 50 μL/mL. Cultures were maintained under standard conditions until reaching 70–80 % confluency [27]. Subsequently, the medium was replaced with RPMI 1640 supplemented with 0.2 % FBS to reduce exogenous growth factors. After 24 h of incubation, the conditioned medium was collected, centrifuged to remove debris, filtered through a 0.22 μm sterile membrane, and stored at —80 °C for future experimental use [28–30].

2.3. Monocytes isolation and purification

Monocytes were isolated from human peripheral blood mononuclear cells (PBMCs) of anonymous healthy blood donors. To obtain PBMCs, blood was diluted in PBS, added carefully to Histopaque Ficoll (d = 1.077, Sigma-Aldrich, Germany), and then centrifuged at 400 × *g* for 20 min at room temperature. Thereafter, the PBMC rings were collected, resuspended and washed twice with PBS by centrifugation at 400 × *g* for 10 min at room temperature [31]. The pellet was suspended in 1 mL of RPMI 1640 for cell counting and testing for viability. Viable cells numbering was assessed by Neubauer chamber on light microscopy (Zeiss, Germany) using Trypan Blue Exclusion Test (TBET) [32]. Finally, monocytes were isolated from PBMCs thanks to adherence method [31,33]. For the assay, PBMCs were cultured in RPMI-1640, supplemented with 10 % FBS and 50 μg gentamicin, placed in a 24-well plate, and then incubated for 1 h at 37 °C and 5 % CO_2 to allow monocyte cells adhesion. The purity of monocytes exceeded over 90 % as routinely evaluated.

2.4. Monocytes activation

For $_{i}$TAG, $_{tcc}$CHOL, H_2O_2, NO, $_{if}Ca^{2+}$ assays, monocytes were activated by 50 U/mL IFN-γ [34], whereas, for NLRP3, NF-kB, LDs, and CD14 and CD16 expression assays, monocytes were activated by 50 U/mL IFN-γ plus 10 ng/mL LPS [35].

2.5. Monocytes treatment

2×10^5 activated monocytes/mL were treated with 1 mM metformin and exposed to a 1:1 ratio of TCM [28], followed by incubation for 24 h at 37 °C and 5 % of CO_2 [34].

2.6. Monocytes lysis

For $_{i}$TAG, $_{tcc}$CHOL and $_{if}Ca^{2+}$ analyses, monocytes were lysed by adding 500 μL of 0.1 % Triton X 100 for 30 min. The reaction was stopped by adding Tris-HCl and $MnCl_2$ mixture [36].

2.7. Functional phenotypic activities assays

2.7.1. Respiratory burst

The respiratory burst was assessed by determining the production levels of H_2O_2 [37–39] and NO [40–42].

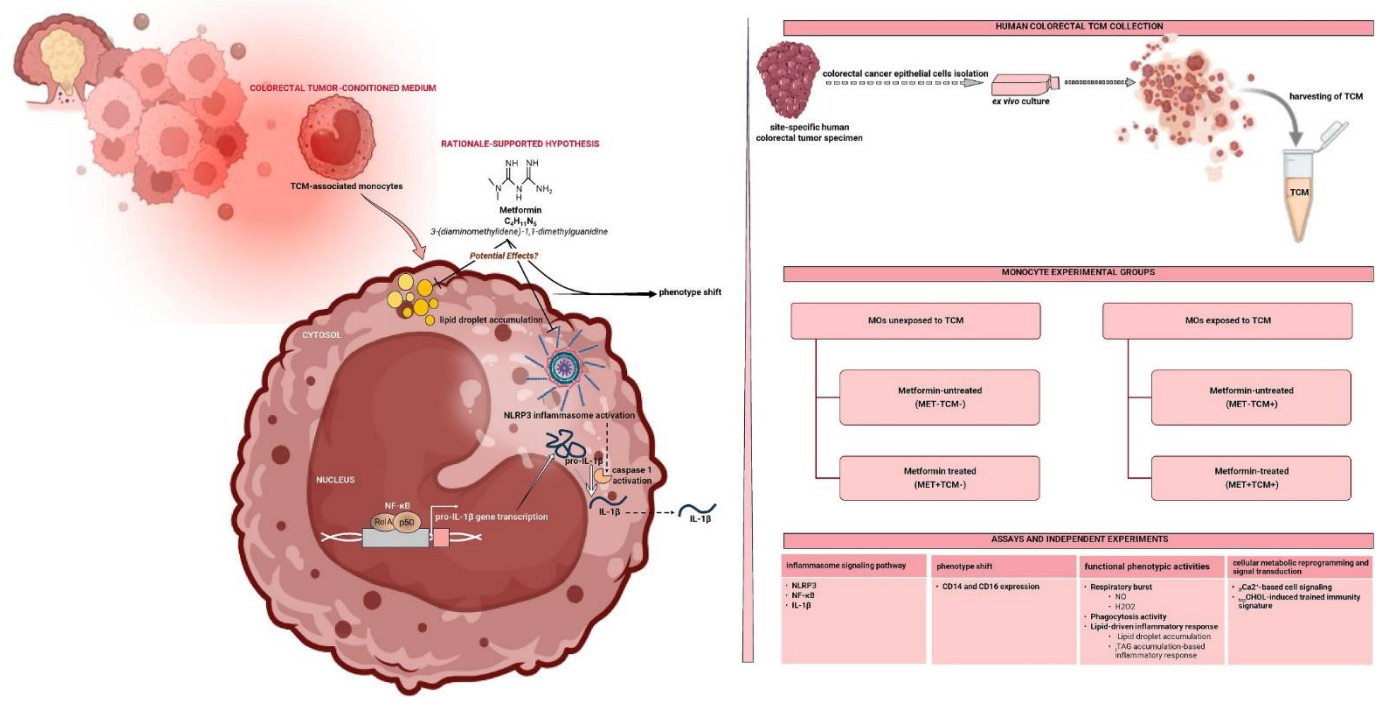

Fig. 1. Study design and experimental workflow. This study aimed to investigate the capacity of metformin to counteract immunometabolic reprogramming induced by colorectal tumor-conditioned medium (TCM) in human monocytes. Primary monocytes were divided into four experimental groups based on their exposure to metformin (MET) and/or TCM: (i) MET—TCM— (metformin-untreated and TCM-unexposed control), (ii) MET+TCM— (metformin-treated and TCM-unexposed), (iii) MET—TCM+ (metformin-untreated and TCM-exposed), and (iv) MET+TCM+ (metformin-treated and TCM-exposed). The study focused on four interconnected axes of analysis: (a) inflammatory signaling, examining NF-κB p65/RelA and NLRP3 expression and IL-1β secretion; (b) phenotypic shift, assessing CD14/CD16 monocyte subset distribution, (c) functional phenotypic activities, (d) cellular metabolic reprogramming and signal transduction. CD14: cluster of differentiation 14, CD16: cluster of differentiation 16, H_2O_2: hydrogen peroxide, $_{if}Ca^{2+}$: intracellular free calcium ions, IL-1β: interleukin-1 beta, $_{i}$TAG: intracellular triacylglycerols, NF-κB p65/RelA: nuclear factor kappa-light-chain-enhancer of activated B cells subunit p65 (RelA), NLRP3: NOD-like receptor family pyrin domain containing 3, NO: nitric oxide, $_{tcc}$CHOL: total cellular cholesterol content.

2.7.1.1. NO assay. NO production levels were determined on the supernatants based on the sensitive Griess colorimetric reaction using a commercial kit (nitric oxide colorimetric assay Roche applied science, Germany) [43]. Absorbance was read at 550 nm with an enzyme-linked immunosorbent assay (ELISA) plate reader (Biochrom Anthos 2020, Cambridge, United Kingdom). The concentration of nitrite was determined according to a standard curve constructed using a solution of sodium nitrite ($NaNO_2$).

2.7.1.2. H_2O_2 assay. H_2O_2 levels were spectrophotometrically measured at 610 nm as described in detail [44]. Briefly, the method was based on the use of a buffered Phenol Red Solution (PRS) containing a peroxide assay buffer (PAB), 0.28 mM (0.1 g/L) of phenol red (phenolsulfonphthalein) and 8.5 U/L (50 mg/mL) of horseradish peroxidase (HRPO, EC 1.11.1.7). Thereafter, cell lysates were added to the assay mixture at a ratio of 1:4 and then incubated for 30 min at 37 °C. The reaction was stopped by adding 10 μL 1 N NAOH. Absorbance was determined against a blank containing PRS and NAOH at the appropriate concentrations. A standard curve was prepared using a dilutions of 30 % H_2O_2 [45].

2.7.2. ROS-dependent NBT-based functional phagocytosis assay

The assay was based on nitro-blue tetrazolium (NBT) method [46], which serves as an indirect indicator of phagocytosis by detecting intracellular ROS generated during the respiratory burst. In brief, 100 μL of NBT (Sigma-Aldrich, Germany) was added to an equal volume 100 μL of cell suspension. Next, the mixture was successively incubated at 37 °C for 15 min, then at room temperature for 15 min. The percentage of cells with intracellular blue-black formazan deposits was assessed under a light microscope equipped with a video camera (Optika, Ponteranica BG, Italy). The phagocytosis percentage was calculated as the ratio of NBT-positive cells to total number of cells [47–49].

2.8. Lipid-driven inflammatory response

2.8.1. Lipid droplet accumulation assay

Lipid droplet accumulation were analyzed using Oil Red O (ORO) [50], a lipophilic diazo dye, commonly used for the staining of neutral lipids, including, triacylglycerols and cholesterol esters [51]. Briefly, 1 × 10^5 monocytes were seeded in a 24-well plate, and rinsed twice with cold 0.01 M PBS, and fixed with 4 % paraformaldehyde for 10 min. The cells were then washed once with PBS for 1 min, and rinsed with 60 % isopropanol for 15 s to enhance lipid staining. Freshly filtered ORO solution was added followed by a dark incubation at 37 °C for 1 min. Excess dye was removed by destaining with 60 % isopropanol for 15 s, and cells were washed thrice with PBS, each lasting 3 min [52,53]. LD accumulation was assessed using Floid Cell Imaging Station (Thermo Fischer Scientific, MA USA), and analyzed by ImageJ/FIJI software (the National Institutes of Health, Bethesda, MD, USA) [54].

2.8.2. $_{i}$TAG accumulation-based inflammatory assay

$_{i}$TAG levels were measured in cell lysates as an essential activator of inflammatory macrophages [55,56] using a spectrophotometric enzymatic method based on triglycerides-derived glycerol phosphate dehydrogenase (GPO)-peroxidase (POD) reactions. This approach produces a red colour formation measurable at 505 nm. $_{i}$TAG levels are expressed in μg/mg protein.

2.9. $_{if}Ca^{2+}$-based cell signaling and activation assay

$_{if}Ca^{2+}$ levels were spectrophotometrically determined at 570 nm by using the orthocresolphthalein complexone (o-CPC) method [57]. Levels of $_{if}Ca^{2+}$ were expressed as μg/mg proteins.

2.10. $_{tcc}$CHOL-based trained immunity assay

The levels of $_{tcc}$CHOL-based innate immune memory/trained immunity [58,59], were spectrophotometrically measured at 505 nm based on Trinder end-point reaction on cell lysates by an enzymatic reaction [60].

2.11. Inflammasome signaling pathway assay

NLRP3, NF-κB p65/RelA, and IL-1β are closely interconnected. NF-κB activates the expression of NLRP3, which forms the inflammasome upon stimulation, triggering IL-1β maturation and release, driving the inflammatory response [61]. Their combined assessment was therefore deemed essential to demonstrate the sequential and interdependent steps of this signaling axis.

2.11.1. Preparation of monocytes for immunofluorescence assays

After TCM exposure and metformin treatment, 1×10^5 monocytes were washed 3 times with PBS, then fixed with 4 % paraformaldehyde for 10 min at 37 °C, followed by a permeabilization with 0.1 % Triton X-100 for 15 min at room temperature. Cells were then blocked with 2 % BSA in PBS for 60 min [62].

2.11.2. NLRP3 and NF-κB p65/RelA expression assays

Monocytes were incubated for 3 h at room temperature with a human NLRP3 antibody conjugated to Alexa Fluor® 647 (dilution 1:50, IC67892R, R&D System), human anti-NF-κB p65/RelA rabbit monoclonal antibody conjugated to Alexa Fluor® 488 (dilution 1:50, ab204263, Abcam, Cambridge, UK). Stained cells were assessed using Floid Cell Imaging Station (Thermo Fischer Scientific, MA USA), and analyzed using FIJI/ImageJ software (the National Institutes of Health, Bethesda, MD, USA).

2.11.3. IL-1β production assay

After 24 h of metformin treatment and TCM exposure, levels of IL-1β were measured in monocytes supernatant by a quantitative sandwich ELISA, then measured using respective human ELISA kits (Sigma Aldrich Co., St. Louis, MO, USA).

2.12. Monocyte subtypes assay

After fixation and blocking, cells were stained with PE-conjugated mouse antihuman CD14 (1:750 dilution, cloneMEM-18 by Sigma-Aldrich), fluorescein isothiocyanate (FITC)-labeled mouse antihuman CD16 (1:200 dilution, clone 3G8 by BD). Fluorescence was assessed using Floid Cell Imaging Station, and analyzed using CellProfiler (version 4.2.8, Broad Institute, Cambridge, MA, USA) to quantify the percentage of $CD14^+$, $CD16^+$, and $CD14^+CD16^+$ cells [63]. Following this analysis, FIJI/ImageJ software was used to quantify corrected total cell fluorescence (CTCF) [64].

2.13. Statistical analyses

Data were analyzed using SPSS version 21 for windows (IBM Corp., Armonk, NY, USA). Normality was assessed using the Shapiro-Wilk test. Comparisons between two groups were performed using the independent Student's t-test. For comparisons involving all groups, one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test was applied. The chi-square (χ^2) test was used to analyze categorical variables, including CD14 and CD16 expression. Data are presented as mean ± standard error of the mean (SEM), and a p-value <0.05 was considered statistically significant.

3. Results

3.1. Metformin downregulates TCM-induced NO and H_2O_2 production

As shown in Fig. 2, monocytes exposed to TCM exhibited significantly upregulated production of both NO and H_2O_2 compared to un- exposed controls (for both, $p < 0.001$ by Student's t-test). However, metformin treatment in the presence of TCM markedly downregulated NO and H_2O_2 levels relative to TCM-exposed, untreated monocytes (NO; $p < 0.05$, H_2O_2; $p < 0.01$ by Student's t-test). Additionally, metformin had no significant effect on NO or H_2O_2 production under basal (non- TCM) conditions ($p > 0.05$). For all comparisons, p-value were less than 0.01 for NO and less than 0.001 for H_2O_2, as determined by one-way ANOVA test.

3.2. Metformin does not reverse TCM-induced impairment of ROS-dependent phagocytosis activity

As depicted in Fig. 3, the phagocytic activity was significantly decreased in both metformin-treated and untreated TCM-exposed monocytes compared to controls ($p < 0.05$ by Student's t-test). However, no significant difference was observed between the TCM-treated and untreated groups ($p > 0.05$ for the two comparisons). According to the one-way ANOVA, all comparisons yielded p-values below 0.01.

3.3. Metformin downregulates TCM-induced intracellular free Ca^{2+} levels

As illustrated in Fig. 4, exposure to TCM alone led to a significant increase in $_{if}Ca^{2+}$ levels ($p < 0.001$). $_{if}Ca^{2+}$ levels in monocytes were significantly downregulated in metformin-treated monocytes compared to untreated controls. This effect was observed both in monocytes treated with metformin alone and in those co-treated with metformin and exposed to TCM ($p < 0.01$, Student's t-test). Additionally, multiple comparisons revealed strong statistical significance ($p < 0.001$), as determined by one-way ANOVA.

3.4. Metformin downregulates TCM-induced $_{tcc}$CHOL accumulation

As shown in Fig. 5, $_{tcc}$CHOL levels were significantly upregulated in the TCM-exposed monocytes compared to controls ($p < 0.001$). Metformin treatment, however, markedly downregulated $_{tcc}$CHOL accumulation relative to metformin-untreated, TCM-exposed monocytes ($p < 0.01$ by Student's t-test). Statistical analysis by one-way ANOVA yielded highly significant differences between groups ($p < 0.0001$).

3.5. Metformin downregulates TCM-induced $_{i}$TAG and lipid droplet accumulation

As shown in Fig. 6, monocytes exposure to TCM resulted in a significant increase in both lipid droplet accumulation and $_{i}$TAG levels compared to the control group (for both, $p < 0.05$ by Student's t-test). However, metformin not only reversed the TCM-induced effects but further drove both variables below baseline values (lipid droplet accumulation; $p < 0.0001$, $_{i}$TAG; $p < 0.001$). For multiple comparisons assessed by one-way ANOVA, p-values were less than 0.0001 for both lipid droplet accumulation accumulation and $_{i}$TAG.

3.6. Metformin downregulates TCM-induced NF-κB p65/RelA and NLRP3 expression, and IL-1β production

As shown in Fig. 7, the expression levels of NF-κB p65/RelA and NLRP3, as well as IL-1β production, were markedly upregulated in TCM-

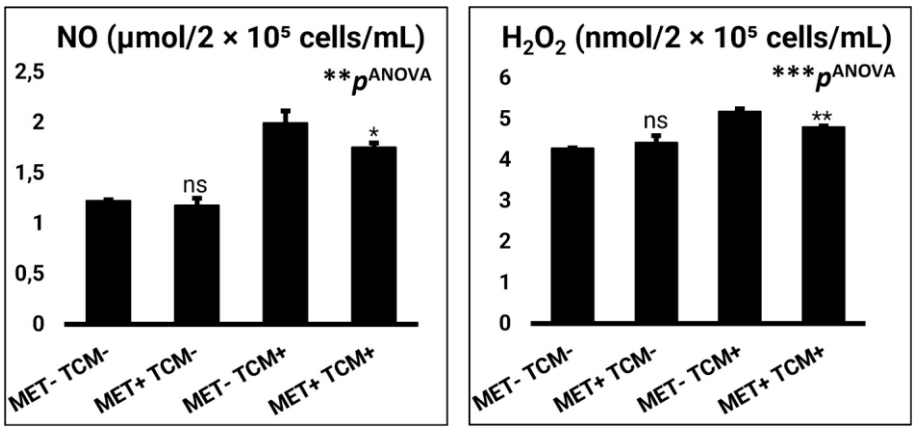

Fig. 2. Metformin and TCM effects on NO and H_2O_2 production. NO and H_2O_2 levels were quantified using specific spectophotometric assays. Values are presented as mean ± standard error of the mean (SEM) from four independent experiments conducted for each of the four groups, using samples from three healthy donors without pooling (n = 12/group). MET: metformin, TCM: tumor-conditioned medium, NO: nitric oxide, H_2O_2: hydrogen peroxide. Monocytes were assigned to four experimental groups: MET—TCM— (metformin-untreated and TCM-unexposed), MET+TCM— (metformin-treated and TCM-unexposed), MET—TCM+ (metformin-untreated and TCM-exposed), and MET+TCM+ (metformin-treated and TCM-exposed). Statistical significance between each pair of groups (MET—TCM— *vs.* MET+TCM— and MET—TCM+ *vs.* MET+TCM+) was assessed using Student's *t*-test, while comparisons among all four groups were performed using one-way analysis of variance (ANOVA). Significant differences are indicated by asterisks. *p < 0.05, **p < 0.01, ***p < 0.001; ns = not significant.

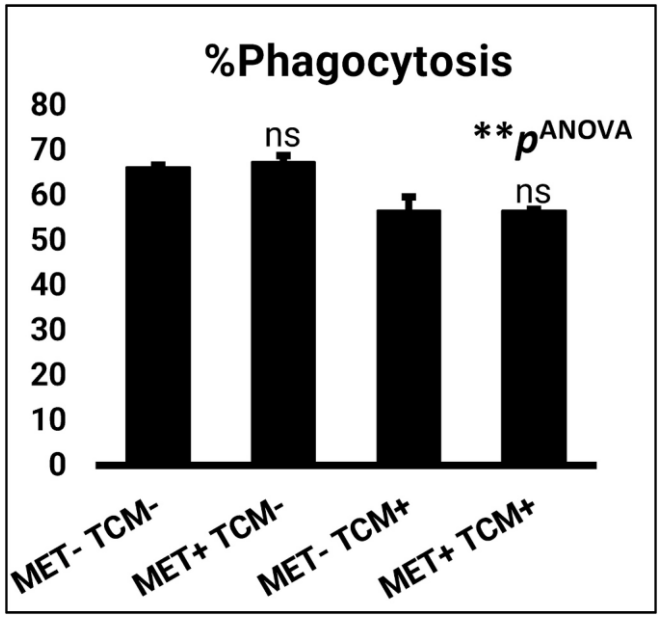

Fig. 3. Metformin and TCM effect on phagocytosis. Phagocytic activity was assessed using a reactive oxygen species (ROS)-dependent nitroblue tetrazolium (NBT) reduction assay. Values are presented as mean ± standard error of the mean (SEM) from four independent experiments conducted for each of the four groups, using samples from three healthy donors without pooling (n = 12/ group). MET: metformin, TCM: tumor-conditioned medium. Monocytes were assigned to four experimental groups: MET—TCM— (metformin-untreated and TCM-unexposed), MET+TCM— (metformin-treated and TCM-unexposed), MET—TCM+ (metformin-untreated and TCM-exposed), and MET+TCM+ (metformin-treated and TCM-exposed). Statistical significance between each pair of groups (MET—TCM— *vs.* MET+TCM— and MET—TCM+ *vs.* MET+TCM+) was assessed using Student's *t*-test, while comparisons among all four groups were performed using one-way analysis of variance (ANOVA). Significant differences are indicated by asterisks. **p < 0.01; ns = not significant.

exposed monocytes compared to controls ($p < 0.001$, $p < 0.01$, and $p < 0.0001$, respectively, by Student's *t*-test). Of note, metformin treatment reversed these effects, downregulating NF-κB and NLRP3 expression and IL-1β production compared to TCM-exposed but untreated monocytes (p

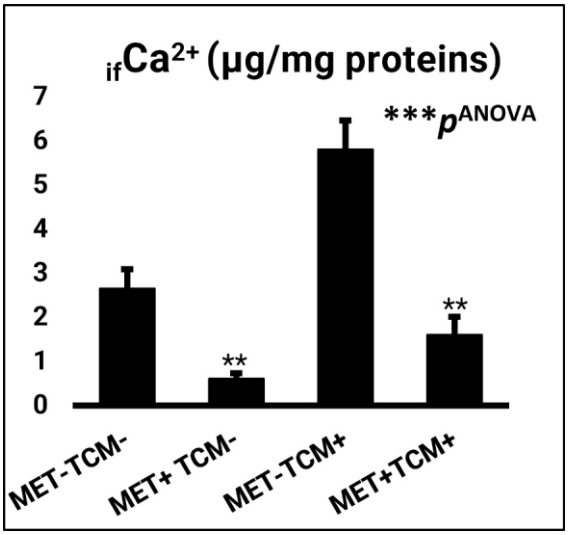

Fig. 4. Metformin and TCM effect on $_{if}Ca^{2+}$. $_{if}Ca^{2+}$ levels were spectrophotometrically determined using the o-Cresolphthalein Complexone (o-CPC) method. Values are presented as mean ± standard error of the mean (SEM) from four independent experiments conducted for each of the four groups, using samples from three healthy donors without pooling (n = 12/group). ifCa2+: intracellular free calcium ions, MET: metformin, TCM: tumor-conditioned medium. Monocytes were assigned to four experimental groups: MET—TCM— (metformin-untreated and TCM-unexposed), MET+TCM— (metformin-treated and TCM-unexposed), MET—TCM+ (metformin-untreated and TCM-exposed), and MET+TCM+ (metformin-treated and TCM-exposed). Statistical significance between each pair of groups (MET—TCM— vs. MET+TCM— and MET—TCM+ vs. MET+TCM+) was assessed using Student's t-test, while comparisons among all four groups were performed using one-way analysis of variance (ANOVA). Significant differences are indicated by asterisks. **p < 0.01, ***p < 0.001 < 0.0001 for all comparisons by Student's t-test). For all comparisons, p-values were less than 0.0001 using one-way ANOVA.

3.7. Metformin modulates monocyte phenotype by restoring CD14 expression and reducing CD16 levels in TCM-exposed monocytes

As demonstrated in Fig. 8, exposure to TCM alone led to a marked

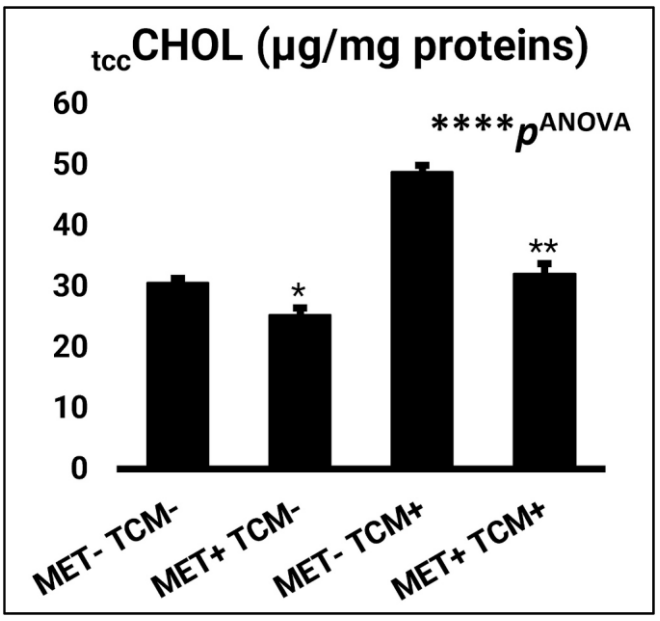

Fig. 5. Effect of Metformin and TCM on $_{tcc}$CHOL. Total cellular cholesterol content ($_{tcc}$CHOL) levels were measured with spectrophotometric method after conversion of cholesterol esters to free cholesterol by the enzyme cholesterol ester hydrolase (EC 3.1.1.1.13). Values are presented as mean ± standard error of the mean (SEM) from four independent experiments conducted for each of the four groups, using samples from three healthy donors without pooling (n = 12/group). MET: metformin, TCM: tumor-conditioned medium. Monocytes were assigned to four experimental groups: MET—TCM— (metformin-untreated and TCM-unexposed), MET+TCM— (metformin-treated and TCM-unexposed), MET—TCM+ (metformin-untreated and TCM-exposed), and MET+TCM+ (metformin-treated and TCM-exposed). Statistical significance between each pair of groups (MET—TCM— *vs.* MET+TCM— and MET—TCM+ *vs.* MET+TCM+) was assessed using Student's *t*-test, while comparisons among all four groups were performed using one-way analysis of variance (ANOVA). Significant differences are indicated by asterisks. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

downregulation of CD14 expression ($p < 0.001$) and a pronounced reduction in CD14$^+$ monocytes ($p < 0.0001$), along with a strong upregulation of CD16 expression ($p < 0.0001$) and an increased proportion of CD16$^+$ monocytes ($p < 0.0001$), indicating a shift toward a CD14^{low}/CD16^{high} proinflammatory phenotype. Importantly, metformin exerted bidirectional control over monocyte surface markers across experimental conditions. Under basal conditions (*i.e.*, in the absence of TCM exposure), metformin significantly reduced both CD14 expression levels ($p < 0.05$) and the proportion of CD14$^+$ monocytes ($p < 0.01$), while significantly increasing CD16 expression ($p < 0.05$) and the frequency of CD16$^+$ monocytes ($p < 0.001$). Under TCM exposure, metformin treatment significantly reversed TCM-induced effects, increasing CD14 expression ($p < 0.05$) and the percentage of CD14$^+$ monocytes ($p < 0.01$), while concurrently decreasing CD16 expression ($p < 0.05$) and the proportion of CD16$^+$ monocytes ($p < 0.01$). For all multiple comparisons assessed by one-way ANOVA, *p*-values were < 0.0001 for CD14 and CD16 expression levels and their respective cell populations, < 0.05 for CD14/CD16 co-expression, and < 0.001 for the frequency of CD14$^+$CD16$^+$ double-positive monocytes.

4. Discussion

This study investigated metformin's capacity to counteract colorectal tumor-conditioned medium-induced immunometabolic dysregulation in monocytes, focusing on three interconnected axes: (a) inflammatory signaling (NF-κB p65/RelA/NLRP3/IL-1β), (b) immuno- metabolic rewiring (lipid droplet accumulation, intracellular triacylglycerol, total cholesterol content and respiratory burst), and (c) phenotypic polarization (CD14/CD16 subsets). Building on evidence that colorectal cancer-derived soluble factors skew monocyte function toward pro-tumorigenic phenotypes, we demonstrated that metformin:
suppressed TCM-driven inflammatory cascades, abrogating NF-κB p65/RelA and NLRP3 upregulation, and IL-1β production, (ii) restored selected immunometabolic homeostasis, reversing TCM-induced lipid droplet, intracellular triacylglycerol, and total cellular cholesterol content accumulation, while modulating respiratory burst biomarkers (nitric oxide, hydrogen peroxide), (iii) reprogrammed monocyte subsets, attenuating the TCM-induced CD14^{low},CD16^{high} shift and partially restoring baseline CD14/CD16 expression patterns.

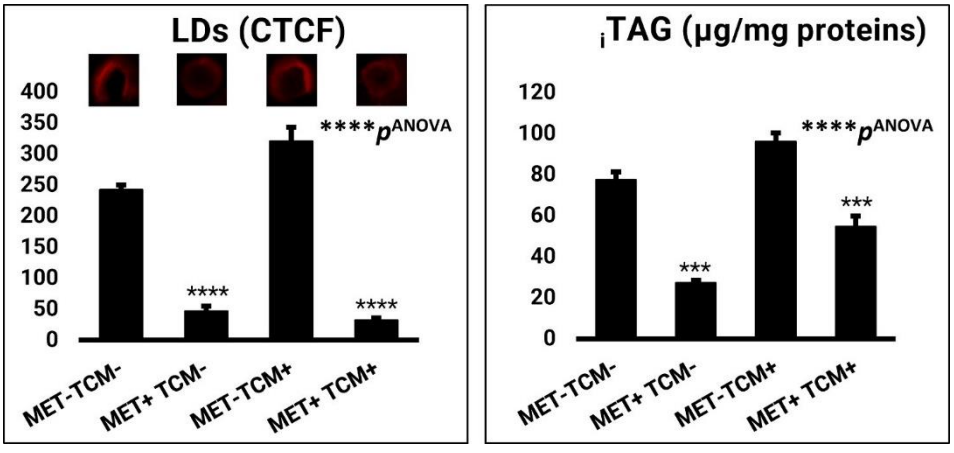

Fig. 6. Effect of Metformin and TCM on $_i$TAG and lipid droplet accumulation. Intracellular triacylglycerol ($_i$TAG) levels were measured using a spectrophotometric method based on enzymatic reactions. Lipid droplet (LD) accumulation was assessed using Oil Red O (ORO) staining, and fluorescence levels were quantified by calculating the corrected total cell fluorescence (CTCF) using ImageJ/FIJI software. Values are presented as mean ± standard error of the mean (SEM) from four independent experiments conducted for each of the four groups, using samples from three healthy donors without pooling (n = 12/group). MET: metformin, TCM: tumor-conditioned medium. Monocytes were assigned to four experimental groups: MET—TCM— (metformin-untreated and TCM-unexposed), MET+TCM— (metformin-treated and TCM-unexposed), MET—TCM+ (metformin-untreated and TCM-exposed), and MET+TCM+ (metformin-treated and TCM-exposed). Statistical significance between each pair of groups (MET—TCM— *vs.* MET+TCM— and MET—TCM+ *vs.* MET+TCM+) was assessed using Student's *t*-test, while comparisons among all four groups were performed using one-way analysis of variance (ANOVA). Significant differences are indicated by asterisks. ***$p < 0.001$, ****$p < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

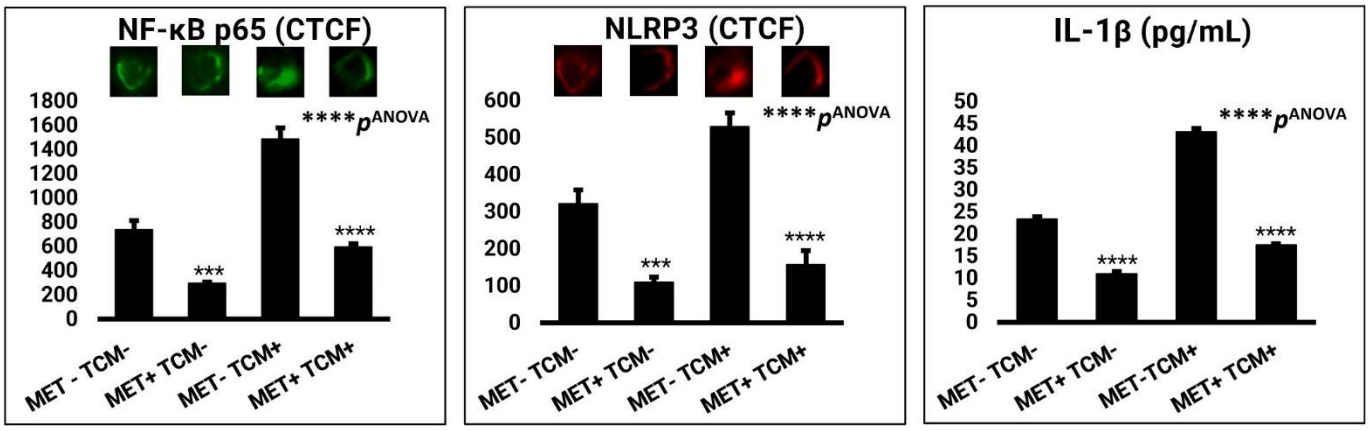

Fig. 7. Effects of metformin and TCM on NF-κB p65/RelA and NLRP3 expression and IL-1β secretion. NF-κB p65/RelA and NLRP3 expression were assessed by immunofluorescent staining using specific fluorophore-conjugated antibodies, and quantified by measuring the corrected total cell fluorescence (CTCF) with ImageJ/FIJI software. Green fluorescence was used to detect NF-κB p65 and red fluorescence for NLRP3. IL-1β secretion levels were determined using a quantitative sandwich enzyme-linked immunosorbent assay (ELISA). Values are presented as mean ± standard error of the mean (SEM) from four independent experiments conducted for each of the four groups, using samples from three healthy donors without pooling (n = 12/group). IL-1β: interleukin-1 beta, MET: metformin, NF-κB p65/RelA: nuclear factor kappa-light-chain-enhancer of activated B cells subunit p65 (RelA), NLRP3: NOD-like receptor family pyrin domain containing 3, TCM: tumor-conditioned medium. Monocytes were assigned to four experimental groups: MET—TCM— (metformin-untreated and TCM-unexposed), MET+TCM— (metformin-treated and TCM-unexposed), MET—TCM+ (metformin-untreated and TCM-exposed), and MET+TCM+ (metformin-treated and TCM-exposed). Statistical significance between each pair of groups (MET—TCM— *vs.* MET+TCM— and MET—TCM+ *vs.* MET+TCM+) was assessed using Student's *t*-test, while comparisons among all four groups were performed using one-way analysis of variance (ANOVA). Significant differences are indicated by asterisks. ***$p < 0.001$, ****$p < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

notably metformin's effects were context-dependent: it exhibited minimal impact under basal conditions but significantly counteracted colorectal tumor-conditioned medium-mediated perturbations, suggesting its actions are preferentially targeted to tumor-modified microenvironments.

4.1. Met effect on respiratory burst in normal conditions and in the presence of TCM

NO and H_2O_2 are key markers of the respiratory burst, reflecting immune cell activation and oxidative stress in inflammatory settings. NO, a gaseous free radical synthesized from L-arginine by nitric oxide synthases (NOS1–3), including the neuronal (nNOS/NOS1), inducible (iNOS/NOS2), and endothelial (eNOS/NOS3) isoforms, is involved in various physiological and pathological processes [65]. In colorectal cancer, TCM containing proinflammatory cytokines may trigger iNOS expression, which may lead to increased NO production. Elevated NO levels may, in turn, promote tumor growth, angiogenesis, and invasiveness, processes linked to poor prognosis [66,67]. H_2O_2 is generated through cellular metabolism and enzymatic activity, notably by nicotinamide adenine dinucleotide phosphate (reduced form, NADPH) oxidase. It contributes to immune responses and signaling, but may also amplify inflammation by stimulating the release of proinflammatory mediators such as high mobility group box 1 protein (HMGB1) [68,69]. Sustained levels of ROS such as H_2O_2 have also been implicated in DNA damage and mutation accumulation, contributing to early colorectal cancer development [70,71].

In our study, monocytes exposed to colorectal cancer-derived TCM exhibited significantly elevated levels of both NO and H_2O_2, indicating an enhanced respiratory burst likely driven by inflammatory signals. This concurrent elevation supports the notion of a TCM-induced oxidative and immuno-inflammatory phenotype in monocytes. Interestingly, treatment with metformin selectively downregulated this response without affecting basal levels. This effect aligns with metformin's known ability to activate AMPK, inhibit NOS2 expression, suppress NADPH oxidase activity, and upregulate antioxidant enzymes such as catalase [72–74]. Moreover, the concurrent downregulation of NO and H_2O_2 by metformin highlights its potential to disrupt pro- tumorigenic signaling within the tumor microenvironment. Prior studies have shown that NO contributes to angiogenesis in colorectal cancer [75], while prolonged H_2O_2 exposure fosters DNA damage and stromal remodeling [76,77]. By quenching both mediators, metformin may deprive tumors of these malignancy-supporting factors. However, given the dual role of ROS in immunity, this effect could theoretically impair cytotoxic functions of monocytes, which rely on ROS to eliminate abnormal cells [78,79]. The functional outcome of this modulation likely depends on the immunological profile of the tumor: in "hot" tumors enriched in effector cells, ROS inhibition might blunt antitumor immunity, whereas in "cold" tumors dominated by proinflammatory and immunosuppressive pathways, it could offer therapeutic benefit.

Altogether, these findings suggest that metformin reshapes monocyte responses to tumor-derived stimuli by modulating the respiratory burst. This mechanism may contribute to metformin's emerging chemopreventive and therapeutic properties in colorectal cancer, although further mechanistic investigations *in vivo* are needed to confirm these observations and define their clinical relevance.

4.2. Metformin effects on TCM-induced immune and inflammatory metabolic reprogramming: focus on $_{tcc}$CHOL, $_{i}$TAG and lipid droplet accumulation

Our findings reveal that colorectal TCM induces marked immunometabolic dysregulation in primary human monocytes, characterized by a significant accumulation of $_{tcc}$CHOL, $_{i}$TAG, and lipid droplets. These alterations reflect a metabolic reorientation toward lipid storage or synthesis, consistent with several reports describing the activation of the phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR) axis in response to tumor-derived cytokines and growth factors [80–82]. This signaling cascade promotes the activity of sterol regulatory element-binding proteins (SREBPs), which enhance *de novo* lipogenesis and cholesterol uptake, while concurrently suppressing cholesterol efflux *via* downregulation of membrane trans- porters involved in cholesterol homeostasis, such as ATP-binding cassette transporter A1 (ABCA1) [83,84].

The reduction of TCM-induced lipid accumulation in monocytes following metformin treatment may reflect its potential to modulate immunometabolic alterations driven by tumor-derived signals. Tumor

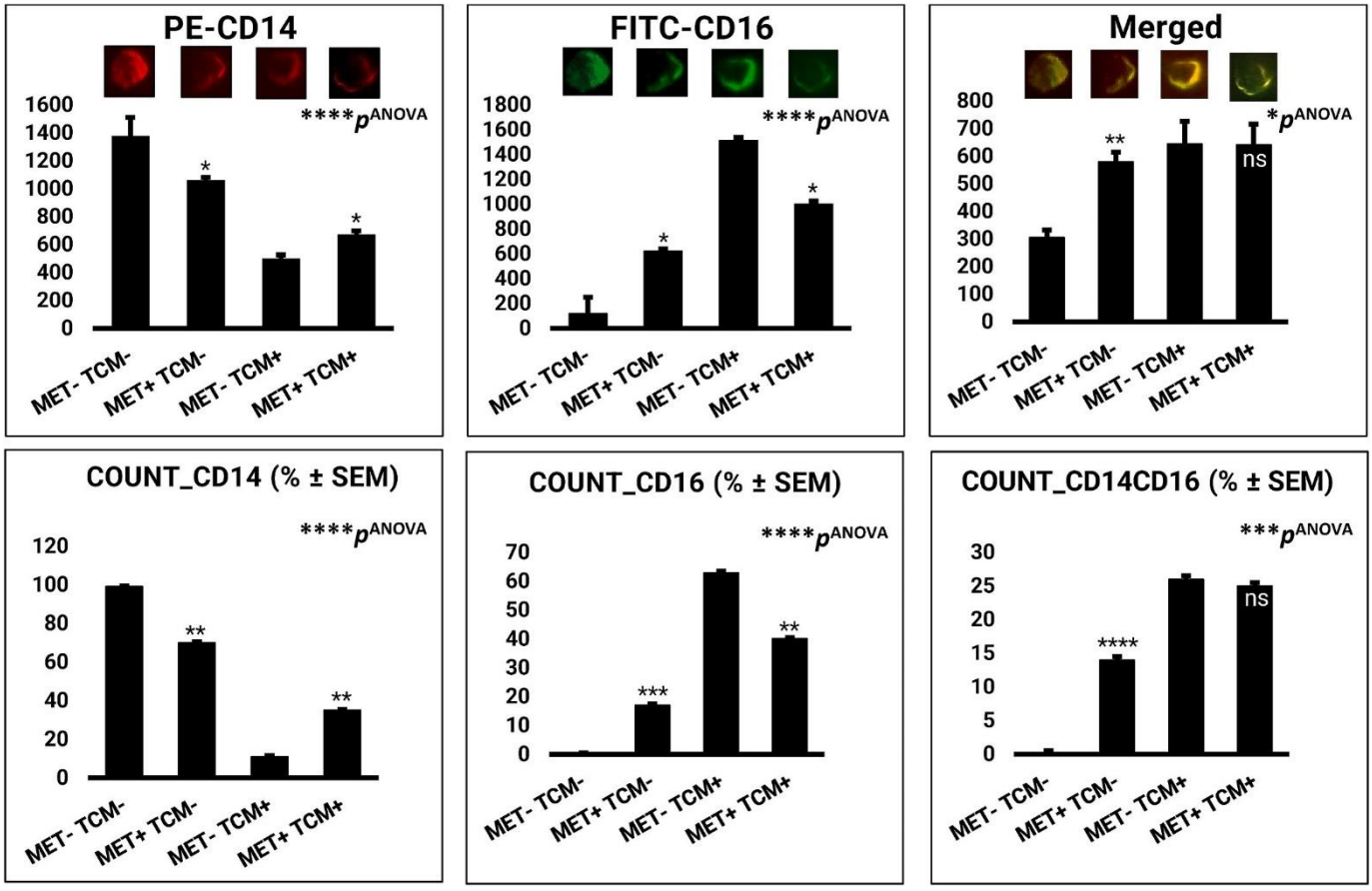

Fig. 8. Effects of metformin and TCM on CD14/CD16 monocyte subsets. Surface expression of CD14 and CD16 was assessed by staining with specific fluorophore-conjugated antibodies. The corrected total cell fluorescence (CTCF) was quantified using ImageJ/FIJI software, and the percentages of $CD14^+$, $CD16^+$, and $CD14^+CD16^+$ monocytes under different experimental conditions were analyzed using CellProfiler. The red fluorescence channel corresponded to CD14 expression, while the green channel corresponded to CD16 expression. Fluorescence imaging was performed using the Floid Cell Imaging Station. Values are presented as mean ± standard error of the mean (SEM) from four independent experiments conducted for each of the four groups, using samples from three healthy donors without pooling (n = 12/group). MET: metformin, PE-CD14: phycoerythrin-conjugated anti-CD14 antibody, FITC-CD16: fluorescein isothiocyanate- conjugated anti-CD16 antibody, TCM: tumor-conditioned medium. Monocytes were assigned to four experimental groups: MET—TCM— (metformin-untreated and TCM-unexposed), MET+TCM— (metformin-treated and TCM-unexposed), MET—TCM+ (metformin-untreated and TCM-exposed), and MET+TCM+ (metformin- treated and TCM-exposed). Statistical significance between each pair of groups (MET—TCM— *vs.* MET+TCM— and MET—TCM+ *vs.* MET+TCM+) was assessed using Student's *t*-test, while comparisons among all four groups were performed using one-way analysis of variance (ANOVA). Significant differences are indicated by asterisks. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; ns = not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

microenvironments typically drive cholesterol and triacylglycerol storage in immune cells, promoting a lipid-laden, dysfunctional state that favors tumor progression [85–87]. Here, metformin not only reversed TCM-driven total cellular cholesterol content and intracellular triacylglycerol ($_{tcc}$CHOL and $_{i}$TAG) accumulation but also reduced lipid droplet accumulation below baseline levels, suggesting that it acts beyond mere normalization, actively depleting lipid stores. Mechanistically, this aligns with metformin's activation of AMPK [88], which inhibits cholesterol synthesis (*via* HMG-CoA reductase phosphorylation) [89] and lipogenesis (through SREBP-1c suppression) [90] while promoting fatty acid oxidation [91]. The sub-baseline reduction in lipid droplet accumulation further implies enhanced lipophagy [92], a process potentiated by AMPK-Unc-51 like autophagy activating kinase 1 (ULK1) signaling [93,94]. This lipid-lowering effect synergizes with metformin's previously noted downregulation of TCM-induced ROS, disrupting a pro-tumorigenic loop where lipid peroxidation fuels oxidative stress.

Beyond metabolic correction, these lipid changes carry functional consequences for monocyte behavior. Lipid droplet accumulation, once thought to be inert storage organelles, are now recognized as active immunometabolic hubs that modulate inflammation and immunity[95,96]. In the context of cancer and chronic inflammation, such lipid- laden monocytes contribute to immune suppression and promote tumor immune evasion [97]. $_{tcc}$CHOL, in particular, has emerged as a key regulator of monocyte epigenetics and trained immunity [98], and our data suggest that the tumor microenvironment may exploit this mechanism to generate a maladaptive trained phenotype favoring tumor growth.

Functionally, depleting lipid reservoirs may restore monocyte anti-tumor potency. Excess cholesterol in TCM-exposed monocytes can amplify oncogenic pathways (*e.g.*, Wingless/Integrated–beta-catenin signaling [Wnt/β-catenin] pathway) [99,100], while lipid droplet accumulation store tumor-promoting mediators like prostaglandins, thromboxanes, and leukotrienes [101–103], they are also essential for cellular immunity, and inhibition of lipid droplet accumulation results in defective cellular immunity [15,104]. By reversing lipid accumulation, metformin likely inhibits protumor signaling and restores phagocytic capacity, countering the immune-paralyzing effects of the TCM. Moreover, the sub-baseline reduction in $_{i}$TAG/lipid droplet accumulation suggests metformin could sensitize monocytes to ferroptosis, an iron-dependent cell death mechanism triggered by lipid peroxidation. This positions metformin as a metabolic checkpoint modulator,

Potentially reversing the lipid-induced "exhaustion" of tumor-infiltrating monocytes. Future studies should explore whether this metabolic reprogramming enhances responses to immunotherapy (*e.g.*, anti–programmed cell death protein 1 [anti-PD-1]), particularly in "cold" tumors where lipid-laden immune cells dominate.

Nonetheless, the therapeutic duality of lipid depletion warrants caution. While impairing protumor functions, excessive lipid loss might compromise energy reserves required for immune activation. Thus, *in vivo* validation is essential to determine whether metformin's lipid-lowering effect ultimately strengthens antitumor immunity or inadvertently dampens monocyte effector responses in colorectal cancer context.

4.3. Metformin and TCM effects on phagocytosis

Phagocytosis, a cornerstone of innate immune defense, is frequently compromised within the tumor microenvironment due to tumor-driven functional and epigenetic reprogramming of monocyte functions [105]. In agreement with this, our data show that exposure to colorectal TCM significantly impairs the phagocytic activity of human monocytes. This dysfunction is consistent with prior reports implicating intracellular lipid overload, oxidative stress, and metabolic exhaustion in the impairment of phagocytic capacity in tumor-associated macrophages (TAMs) [106,107]. Although metformin markedly downregulated TCM-induced $_{tcc}$CHOL, $_{i}$TAG and lipid droplet accumulation, it failed to restore phagocytosis. This observation suggests that lipid dysregulation, although relevant, is not solely responsible for the observed defect. The persistence of this effect, despite correction of key metabolic parameters, reveals a robust TCM-imposed immune mechanism. Tumor-derived factors, including immunosuppressive cytokines and extracellular vescles such as exosomes and microvesicles, are known to induce sustained epigenetic alterations, including DNA methylation, histone modifications, post-transcriptional RNA regulation, and changes in miRNA expression patterns, which are major epigenetic features in colorectal cancer [108,109]. These modifications can lead to the derepression of specific transcriptional programs affecting the phagocytic machinery, as recently observed in post-inflammatory settings [110], notably through the targeting of actin cytoskeleton remodeling pathways [111].

It is entirely possible that metformin's inability to reverse this phagocytic suppression, despite improvements in metabolic indicators, also suggests the involvement of additional immunosuppressive mechanisms. Potential mechanisms may include: (i) dysregulated activation of transcription factors, notably signal transducer and activator of transcription 3 (STAT3) [112], given that metformin has been reported to inhibit STAT3 activity in tumor cells [113]; (ii) the upregulation of inhibitory immune checkpoints, particularly signal regulatory protein-α (SIRPα), a myeloid-specific receptor that mediates the "don't eat me" signal through its interaction with CD47 [114]. While it remains un- known whether soluble or shed forms of CD47 are present in the TCM, or whether an additional agonist capable of activating SIRPα is involved, it is unclear at this stage whether metformin can modulate this axis; and (iii) persistent metabolic dysfunctions, such as elevated mitochondrial ROS or altered glycolytic flux, which may continue to impair phagocytic competence after metformin treatment [115,116].

Together, these findings highlight a critical therapeutic limitation: metformin, while metabolically reprogramming monocytes, does not suffice to fully restore their phagocytic competence in a TCM. This emphasizes the need for combinatorial strategies. Agents that antagonize phagocytosis-inhibitory pathways or that activate innate immune pathways, like stimulator of interferon genes (STING, also known as transmembrane protein 173 [TMEM173] and MPYS/MITA/ERIS) agonists [117], may act synergistically with metformin to reestablish phagocytic competence and overcome colorectal TCM-mediated immunosuppression.Importantly, metformin did not impair basal phagocytic activity under non-tumoral conditions, consistent with previous findings [118], thereby reinforcing its favorable safety profile and supporting its potential inclusion in multi-agent immunometabolic strategies. However, in the context of colorectal cancer, our data indicate that metformin alone is insufficient to reverse TCM-induced phagocytic dysfunction. As such, it should be regarded as a supportive rather than a stand-alone agent in therapeutic approaches aimed at restoring antitumor innate immune responses.

4.4. Metformin effect on free calcium ions in TCM-exposed monocytes

As a universal and critical second messenger, $_{if}Ca^{2+}$ regulates essential immune cell functions, including phagocytosis [119] (notably by modulating cytoskeletal remodeling *via* calcium-sensitive proteins such as myosin, actin regulators, annexins, calmodulin, and the calcium-activated protease calpain [120–126]), signal transduction, cytokine secretion, and activation [127,128]. In our study, colorectal cancer-derived TCM significantly increased $_{if}Ca^{2+}$ levels in human monocytes. This effect is likely mediated by soluble tumor-derived factors, including cytokines, chemokines, growth factors, small molecule mediators, ATP, and danger signals [129–131], *etc.*, that activate G-protein- coupled receptors (GPCRs) [132] and toll-like receptors (TLRs) [133]. These pathways converge on phospholipase C (PLC) activation and to the subsequent inositol trisphosphate (IP_3) production, leading to calcium release from intracellular stores and influx from the extracellular space through specific membrane channels [134–136]. The tumor- induced calcium mobilization in monocytes is particularly important, as increased intracellular calcium has been associated with their activation and polarization toward an M2-like (characterized by calcium release-activated calcium channel protein 1 [Orai1]-dependent Ca^{2+} influx, unlike M1 cells relying on transient receptor potential canonical 1 [TRPC1]-mediated currents [137]), immunosuppressive phenotype that supports tumor progression [138,139]. However, to our knowledge, no previous study has directly demonstrated such a calcium increase in monocytes exposed to a tumor-conditioned environment, especially in the context of colorectal cancer. This limits direct comparison with existing experimental data, underscoring the novelty of our observations. Nevertheless, our findings are consistent with emerging literature highlighting the critical role of calcium signaling in regulating immune cell behavior within the tumor micronevironment. Several studies have emphasized the involvement of calcium channels and transporters in immune suppression, tumor-associated inflammation, and cancer progression [140–143]. Of great interest, we observed that metformin treatment reversed the TCM-induced increase in intracellular calcium in monocytes. Although we did not directly assess mitochondrial complex I-dependent respiration activity, ATP levels, or AMPK activation in this study, existing literature provides mechanistic insight. Hence, metformin is known to inhibit mitochondrial complex I and mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH) and to activate sirtuin 1 (SIRT1) and SIRT3, leading to decreased ATP production and consequent activation of AMPK [144,145]. AMPK, in turn, can modulate calcium homeostasis by regulating calcium channels, pumps, and signaling intermediates involved in immune cell activation [146,147].

Finally, our results add to a growing body of evidence suggesting that metformin can reprogram immune cells through both metabolic and calcium-dependent mechanisms. These effects may contribute to its broader immunomodulatory and anti-tumoral properties, particularly in reshaping monocyte/macrophage responses within the tumor microenvironment.

4.5. Metformin effect on the NF-κB–NLRP3–IL-1β axis in TCM-exposed monocytes

Our findings reveal that TCM derived from colorectal cancer markedly activates the NF-κB p65/RelA–NLRP3–IL-1β axis in human monocytes, suggesting that these innate immune cells are among the k e y

orchestrators of inflammation within the tumor-conditioned microenvironment. This observation aligns with the established role of NF-κB p65 as a master regulator of inflammation-driven carcinogenesis, where constitutive activation fosters a permissive microenvironment for tumor initiation, progression, and immune evasion [148]. The upregulation of NF-κB p65 in monocytes exposed to colorectal cancer TCM likely stems from soluble tumor-derived factors that engage pattern recognition receptors (PRRs), triggering canonical signaling pathways [149].

Concurrently, NLRP3 inflammasome activation and subsequent IL-1β secretion, a process initiated by NF-κB signaling, in monocytes exposed to colorectal cancer-derived TCM may contribute to a self-amplifying inflammatory loop. Indeed, IL-1β has been shown to stimulate both NF-κB p65 activation and NLRP3 expression in neighboring stromal and immune cells, thereby establishing a feedforward cascade that reinforces inflammation and potentially promotes tumor progression [150]. Mechanistically, NLRP3 activation in colorectal cancer models has been shown to promote epithelial-to-mesenchymal transition (EMT) through the transforming growth factor-beta (TGF-β)/Smad signaling pathway and to facilitate metastatic dissemination, while IL-1β recruits TAMs that polarize toward an M2-like macrophages, pro- angiogenic phenotype [150,151]. This synergy between NF-κB p65 and NLRP3 may underpin the "tumor-promoting inflammation" hall- mark, where monocytes, reprogrammed by TCM, become architects of an immunosuppressive niche.

Our observation that metformin effectively inhibits TCM-induced activation of the NF-κB p65/RelA–NLRP3 inflammasome–IL-1β axis in monocytes introduces a promising immunomodulatory strategy for disrupting tumor-promoting inflammation in colorectal cancer. While the immunopharmacological properties of metformin are well established across various experimental systems, its capacity to reverse proinflammatory signaling in monocytes exposed to colorectal cancer-derived soluble factors has, to our knowledge, yet to be documented, positioning our findings as a novel contribution to the field.

Metformin's inhibition of NLRP3 inflammasome expression in our model mirrors recent findings in alveolar macrophages during severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-induced acute respiratory distress syndrome (ARDS), where it blocks ATP-dependent mitochondrial DNA (mtDNA) synthesis, a critical ligand for NLRP3, independently of AMPK [152]. This AMPK-independent mechanism may account for its efficacy in TCM-exposed monocytes, as colorectal cancer-derived soluble factors could similarly promote mtDNA release, sustaining NLRP3 activation. Moreover, the downregulation of IL-1β observed in our study parallels metformin's effects in chronic stress condition, where it attenuates NLRP3-driven neuroinflammation in both macrophages and hippocampal microglia [153]. These consistent findings suggest that metformin may engage a conserved mechanism to disrupt the feedforward amplification between NF-κB p65 and NLRP3 signaling, potentially across diverse inflammatory contexts. In our study, the drug's ability to inhibit NF-κB p65 expression could also resonate with previously reported mechanisms in vascular inflammation, where AMPK activation inhibits IκB kinase (IKK), thereby pre- venting IκBα degradation and nuclear translocation of p65 [154]. While these pathways remain to be formally confirmed in tumor-conditioned monocytes, such observations may offer mechanistic clues underpinning metformin's immunomodulatory action in the colorectal cancer setting. In TCM-exposed monocytes, metformin likely exploits this pathway to quell NF-κB p65-driven transcription of NLRP3, IL1β, and chemokines that amplify immune dysfunction in colorectal cancer. Notably, metformin's dual targeting of both NF-κB p65 (upstream initiator) and NLRP3 (downstream effector) may confer advantages over single-axis inhibitors. Furthermore, given the central role of IL-1β in shaping the inflammatory crosstalk between immune and stromal cells, metformin's inhibition of its secretion warrants *in vivo* investigation to assess potential indirect effects on the neighboring tumor stroma.

In fine, metformin's ability to neutralize the NF-κB p65–NLRP3–IL-1β axis in TCM-exposed monocytes unveils a new dimension of its immunomodulatory prowess. Harnessing this mechanism could break the cycle of tumor-promoting inflammation, offering a viable adjunct to conventional colorectal cancer therapies.

4.6. Metformin effect on monocyte subset plasticity

Human primary monocytes represent a heterogeneous population classified into three main subsets based on CD14 and CD16 surface expression: classical ($CD14^{++}/CD16^{-}$), intermediate ($CD14^{++}/CD16^{+}$), and non-classical ($CD14^{+}/CD16^{++}$) monocytes [10]. These subsets exhibit distinct functional profiles, particularly in inflammation, tissue surveillance, and antigen presentation.

The plasticity of monocytes in the tumor microenvironment warrants careful consideration. Our data show that exposure to the TCM induced a profound phenotypic shift in monocytes, characterized by marked downregulation of CD14 and strong upregulation of CD16, promoting a $CD14^{low}/CD16^{high}$ phenotype. This reprogramming is consistent with a non-classical, proinflammatory subset [155], which has been associated with elevated NF-κB p65 activity, enhanced ROS production (including NO and H_2O_2), and high levels of IL-1β, as well as reduced phagocytic capacity [149,155,156]. These features suggest that the TCM drives monocytes toward a dysfunctional, tumor-supportive state, contributing to immune evasion and chronic inflammation in the tumor microenvironment.

Importantly, metformin treatment reversed these TCM-induced alterations, significantly restoring CD14 expression while reducing CD16 levels. This shift suggests a reversion toward a more classical or inter- mediate phenotype, generally associated with controlled activation, improved phagocytosis, and a balanced immune response [157]. This phenotype change aligns with our earlier observations of reduced ROS, lower IL-1β production, and dampened NF-κB p65–NLRP3 signaling under metformin treatment.

While metformin's impact on surface marker expression has been primarily explored in metabolic and inflammatory disease contexts [158,159], its ability to reshape monocyte phenotype in a colorectal tumor-conditioned setting provides new insight into its potential as an immunotherapeutic adjuvant. Mechanistically, this phenotypic reprogramming may reflect the observed metformin's capacity to restore metabolic homeostasis and epigenetic integrity in immune cells exposed to various inflammatory conditions, including tumor-derived metabolic cues [160–165]. Indeed, TCM-driven skewing toward a $CD14^{low}/CD16^{high}$ subset may involve metabolic rewiring favoring glycolysis and redox imbalance [166], two axes known to be sensitive to metformin's action on AMPK and mitochondrial function, as mentioned above.

Although direct studies on metformin's effect on monocyte subset phenotypes are limited, our results echo findings in macrophage populations, where metformin was shown to decrease M2-like (tumor-promoting) macrophages and promote M1-like (proinflammatory/anti-tumor) polarization [157]. These parallels suggest that metformin may act as a broader modulator of myeloid cell plasticity, including both circulating and tissue-resident compartments.

Together, these findings highlight metformin's capacity to restore a monocyte phenotype more compatible with anti-tumor immunity, notably by reversing TCM-induced functional alterations. This phenotypic reprogramming may also favor the recruitment and activation of key immune effectors, such as dendritic cells and cytotoxic lymphocytes, thereby contributing to a more robust and coordinated anti-tumor response. These results further support metformin's therapeutic repositioning within oncological immunomodulation strategies.

5. Conclusions and future prospects

This study provides compelling evidence that metformin exerts multifaceted regulatory effects on monocytes exposed to colorectal TCM, reversing immunometabolic and inflammatory dysregulation. Specifically, metformin modulated TCM-induced respiratory burst,

intracellular free calcium ions, and selected immunometabolites accumulation. These effects are suggestive of a broader immunometabolic reprogramming favoring homeostasis.

Furthermore, metformin markedly modulated key components of the NF-κB p65/NLRP3/IL-1β axis, a major signaling pathway implicated in chronic inflammation and tumor progression. This modulatory effect was accompanied by phenotypic reprogramming of monocyte subsets, notably reversing the TCM-induced shift toward a proinflammatory $CD14^{low}/CD16^{high}$ profile and partially restoring the expression of classical monocyte markers. Of note, under basal conditions, metformin had minimal effects, reinforcing the notion of its context-dependent activity, primarily targeting tumor-modified or inflamed microenvironments.

Despite these promising findings, metformin did not rescue the TCM-induced impairment of ROS-dependent phagocytic capacity, indicating that not all functional aspects of monocyte dysregulation are reversible by metformin alone. This underscores the selectivity and limitations of its modulatory effects and highlights the complexity of tumor–immune interactions.

Taken together, the results suggest that metformin, beyond its canonical antidiabetic role, possesses significant immunomodulatory potential in the context of tumor-associated inflammation. Its ability to selectively restore metabolic and phenotypic homeostasis in monocytes exposed to tumor-derived factors may hold translational relevance, especially in designing adjunctive strategies for cancer immunotherapy or inflammation-targeted interventions.

These findings open several avenues for future investigation. First, *in vivo* validation using preclinical models of colorectal cancer is warranted to confirm the observed protective effects of metformin within a more complex immunological and tumorigenic landscape. Second, investigating whether the suppression of lipid accumulation and inflammasome activation involves AMPK signaling could yield novel therapeutic targets, as suggested by prior studies in related contexts.

Moreover, it would be of interest to explore whether combining metformin with other immunometabolic modulators or checkpoint inhibitors could achieve synergistic anti-tumoral immune reprogramming. Longitudinal studies evaluating metformin's impact on myeloid-derived suppressor cells (MDSCs), dendritic cells, and macrophages in the tumor microenvironment could also extend the scope of its application.

Finally, clinical investigations into metformin's off-label use in non-diabetic cancer patients, particularly those exhibiting high monocyte-driven inflammation or lipid-rich immune phenotypes, may help to stratify responsive subpopulations and pave the way toward precision immunometabolic therapy.

CRediT authorship contribution statement

Nihel Chahinez Djebri: Writing – original draft, Visualization, Software, Investigation, Formal analysis. **Souad Zoudji:** Writing – review & editing, Validation, Investigation, Data curation. **Aida Messaoud:** Writing – review & editing, Resources, Methodology. **Rabia Messali:** Writing – review & editing, Resources, Project administration, Investigation. **Salim Loudjedi:** Writing – review & editing, Visualization, Supervision, Software, Conceptualization. **Mourad Aribi:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Software, Funding acquisition, Conceptualization.

Author statement

N.C.D. performed most of the experimental work. S.Z., A.M., and R. M. contributed to the laboratory experiments, data acquisition, and analysis. S.L. provided site-specific tumor specimens, offered clinical insight, and contributed to the interpretation of results. M.A. conceptualized and designed the study, supervised the scientific work at all stages, provided critical intellectual input, secured funding, coordinated the research team, and ensured the overall coherence and scientific integrity of the project. M.A. and N.C.D. wrote the original draft of the manuscript. All authors critically revised and approved the final version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

References

[1] C.W.-K. Wu, R.N. Lui, Early-onset colorectal cancer: current insights and future directions, world, J. Gastrointest. Oncol. 14 (2022) 230–241, https://doi.org/10.4251/wjgo.v14.i1.230.

[2] A. Hnatyszyn, S. Hryhorowicz, M. Kaczmarek-Ry´s, E. Lis, R. Słomski, R.J. Scott, A. Pławski, Colorectal carcinoma in the course of inflammatory bowel diseases, Hered. Cancer Clin. Pract. 17 (2019) 18, https://doi.org/10.1186/s13053-019-0118-4.

[3] Y. Zhang, Y. Zhao, Q. Li, Y. Wang, Macrophages, as a promising strategy to targeted treatment for colorectal Cancer metastasis in tumor immune microenvironment, Front. Immunol. 12 (2021) 685978, https://doi.org/10.3389/fimmu.2021.685978.

[4] J.M. Pitt, A. Marabelle, A. Eggermont, J.-C. Soria, G. Kroemer, L. Zitvogel, Targeting the tumor microenvironment: removing obstruction to anticancer immune responses and immunotherapy, Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. 27 (2016) 1482–1492, https://doi.org/10.1093/annonc/mdw168.

[5] S.I. Grivennikov, F.R. Greten, M. Karin, Immunity, inflammation, and cancer, Cell 140 (2010) 883–899, https://doi.org/10.1016/j.cell.2010.01.025.

[6] W.-T. Kuo, J.-M. Chang, C.-C. Chen, N. Tsao, C.-P. Chang, Autophagy drives plasticity and functional polarization of tumor-associated macrophages, IUBMB Life 74 (2022) 157–169, https://doi.org/10.1002/iub.2543.

[7] M. Djaldetti, H. Bessler, Mononuclear cells phagocytic activity affects the crosstalk between immune and cancer cells, Biomed. Pharmacother. Biomedecine Pharmacother. 68 (2014) 679–683, https://doi.org/10.1016/j.biopha.2014.08.004.

[8] J. Kim, Y. Yuan, K. Agaronyan, A. Zhao, V.D. Wang, D. Gau, N. Toosi, G. Gupta, H. Essayas, A. Kaminski, J. McGovern, S. Yu, S. Woo, C.J. Lee, S. Gandhi, T. Saber, T. Saleh, B. Hu, Y. Sun, G. Ishikawa, W. Bain, J. Evankovich, L. Chen, H. Yun, E.L. Herzog, C.S. Dela Cruz, C. Ryu, L. Sharma, Damage sensing through TLR9 regulates inflammatory and antiviral responses during influenza infection, Mucosal Immunol. (2025), https://doi.org/10.1016/j.mucimm.2025.01.008. S1933–0219(25)00008-X.

[9] A. Martin, S. Caron, M. Marcotte, P. Bronnec, E. Garneret, N. Martel, G. Maalouf, P. S`eve, D. Saadoun, Y. Jamilloux, T. Henry, IFN-γ licenses normal and pathogenic ALPK1/TIFA pathway in human monocytes, iScience 28 (2025) 111563, https://doi.org/10.1016/j.isci.2024.111563.

[10] T.S. Kapellos, L. Bonaguro, I. Gemünd, N. Reusch, A. Saglam, E.R. Hinkley, J. L. Schultze, Human monocyte subsets and phenotypes in major chronic inflammatory diseases, Front. Immunol. 10 (2019) 2035, https://doi.org/10.3389/fimmu.2019.02035.

[11] P. Vishnyakova, A. Poltavets, E. Karpulevich, A. Maznina, V. Vtorushina, L. Mikhaleva, E. Kananykhina, A. Lokhonina, S. Kovalchuk, A. Makarov, A. Elchaninov, G. Sukhikh, T. Fatkhudinov, The response of two polar monocyte subsets to inflammation, Biomed. Pharmacother. 139 (2021) 111614, https://doi.org/10.1016/j.biopha.2021.111614.

[12] X. Zhuang, X. Li, J. Zhang, Y. Hu, B. Hu, Y. Shi, Y. Sun, G. Hong, Conditioned medium mimicking the tumor microenvironment augments chemotherapeutic resistance via ataxia-telangiectasia mutated and nuclear factor-κB pathways in gastric cancer cells, Oncol. Rep. 40 (2018) 2334–2342, https://doi.org/10.3892/or.2018.6637.

[13] D. Kwart, J. He, S. Srivatsan, C. Lett, J. Golubov, E.M. Oswald, P. Poon, X. Ye, J. Waite, A.G. Zaretsky, S. Haxhinasto, E. Au-Yeung, N.T. Gupta, J. Chiu, C. Adler, S. Cherravuru, E. Malahias, N. Negron, K. Lanza, A. Coppola, M. Ni, H. Song,

Y. Wei, G.S. Atwal, L. Macdonald, N.S. Oristian, W. Poueymirou, V. Jankovic, M. Fury, I. Lowy, A.J. Murphy, M.A. Sleeman, B. Wang, D. Skokos, Cancer cell-derived type I interferons instruct tumor monocyte polarization, Cell Rep. 41 (2022) 111769, https://doi.org/10.1016/j.celrep.2022.111769.
[14] T.-Y. Lin, M.-C. Tsai, W. Tu, H.-C. Yeh, S.-C. Wang, S.-P. Huang, C.-Y. Li, Role of the NLRP3 Inflammasome: insights into Cancer hallmarks, Front. Immunol. 11 (2020) 610492, https://doi.org/10.3389/fimmu.2020.610492.
[15] A.L.S. Cruz, E.D.A. Barreto, N.P.B. Fazolini, J.P.B. Viola, P.T. Bozza, Lipid droplets: platforms with multiple functions in cancer hallmarks, Cell Death Dis. 11 (2020) 105, https://doi.org/10.1038/s41419-020-2297-3.
[16] C.-A.W. Ng, A.A. Jiang, E.M.S. Toh, C.H. Ng, Z.H. Ong, S. Peng, H.Y. Tham, R. Sundar, C.S. Chong, C.M. Khoo, Metformin and colorectal cancer: a systematic review, meta-analysis and meta-regression, Int. J. Color. Dis. 35 (2020) 1501–1512, https://doi.org/10.1007/s00384-020-03676-x.
[17] L.P. Bharath, B.S. Nikolajczyk, The intersection of metformin and inflammation, Am. J. Phys. Cell Phys. 320 (2021) C873–C879, https://doi.org/10.1152/ajpcell.00604.2020.
[18] T.T. Nguyen, T.T. Ung, S. Li, S. Lian, Y. Xia, S.Y. Park, Y. Do Jung, Metformin inhibits lithocholic acid-induced interleukin 8 upregulation in colorectal cancer cells by suppressing ROS production and NF-kB activity, Sci. Rep. 9 (2019) 2003, https://doi.org/10.1038/s41598-019-38778-2.
[19] M. Moossavi, N. Parsamanesh, A. Bahrami, S.L. Atkin, A. Sahebkar, Role of the NLRP3 inflammasome in cancer, Mol. Cancer 17 (2018) 158, https://doi.org/10.1186/s12943-018-0900-3.
[20] T. Zhang, C. Ma, Z. Zhang, H. Zhang, H. Hu, NF-κB signaling in inflammation and cancer, MedComm 2 (2021) 618–653, https://doi.org/10.1002/mco2.104.
[21] A. Abderrazak, T. Syrovets, D. Couchie, K. El Hadri, B. Friguet, T. Simmet, M. Rouis, NLRP3 inflammasome: from a danger signal sensor to a regulatory node of oxidative stress and inflammatory diseases, Redox Biol. 4 (2015) 296–307, https://doi.org/10.1016/j.redox.2015.01.008.
[22] C.A. Dinarello, Interleukin-1 in the pathogenesis and treatment of inflammatory diseases, Blood 117 (2011) 3720–3732, https://doi.org/10.1182/blood-2010-07-273417.
[23] V. Singh, S. Ubaid, M. Kashif, T. Singh, G. Singh, R. Pahwa, A. Singh, Role of inflammasomes in cancer immunity: mechanisms and therapeutic potential, J. Exp. Clin. Cancer Res. CR 44 (2025) 109, https://doi.org/10.1186/s13046-025-03366-y.
[24] R. Ma, B. Yi, A.I. Riker, Y. Xi, Metformin and cancer immunity, Acta Pharmacol. Sin. 41 (2020) 1403–1409, https://doi.org/10.1038/s41401-020-00508-0.
[25] M. Arul, A.C. Roslani, C.L.L. Ng, S.H. Cheah, Culture of low passage colorectal cancer cells and demonstration of variation in selected tumour marker expression, Cytotechnology 66 (2014) 481–491, https://doi.org/10.1007/s10616-013-9600-4.
[26] A. Zubeldia-Plazaola, E. Ametller, M. Mancino, M. Prats de Puig, A. Lo´pez-Plana, F. Guzman, L. Vinyals, E.M. Pastor-Arroyo, V. Almendro, G. Fuster, P. Gasco´n, Comparison of methods for the isolation of human breast epithelial and myoepithelial cells, Front. Cell Dev. Biol. 3 (2015) 32, https://doi.org/10.3389/fcell.2015.00032.
[27] Y. Zhang, Z. Zhang, L. Chen, X. Zhang, Tumor cells-derived conditioned medium induced pro-tumoral phenotypes in macrophages through calcium-nuclear factor κB interaction, BMC Cancer 22 (2022) 1327, https://doi.org/10.1186/s12885-022-10431-8.
[28] B. Benner, L. Scarberry, L.P. Suarez-Kelly, M.C. Duggan, A.R. Campbell, E. Smith, G. Lapurga, K. Jiang, J.P. Butchar, S. Tridandapani, J.H. Howard, R.A. Baiocchi, T.A. Mace, W.E. Carson, Generation of monocyte-derived tumor-associated macrophages using tumor-conditioned media provides a novel method to study tumor-associated macrophages in vitro, J. Immunother. Cancer 7 (2019) 140, https://doi.org/10.1186/s40425-019-0622-0.
[29] K. Sawa-Wejksza, A. Dudek, M. Lemieszek, K. Kaławaj, M. Kandefer-Szerszen´, Colon cancer-derived conditioned medium induces differentiation of THP-1 monocytes into a mixed population of M1/M2 cells, tumour biol, J. Int. Soc. Oncodevelopmental Biol. Med. 40 (2018), https://doi.org/10.1177/1010428318797880, 1010428318797880.
[30] Y.P. Chong, E.P. Peter, F.J.M. Lee, C.M. Chan, S. Chai, L.P.C. Ling, E.L. Tan, S. H. Ng, A. Masamune, S.A.A. Ghafar, N. Ismail, K.L. Ho, Conditioned media of pancreatic cancer cells and pancreatic stellate cells induce myeloid-derived suppressor cells differentiation and lymphocytes suppression, Sci. Rep. 12 (2022) 12315, https://doi.org/10.1038/s41598-022-16671-9.
[31] L.M. Wahl, S.M. Wahl, L.E. Smythies, P.D. Smith, Isolation of human monocyte populations, Curr. Protoc. Immunol. 70 (2005), https://doi.org/10.1002/0471142735.im0706as70.
[32] W. Strober, Trypan blue exclusion test of cell viability, Curr. Protoc. Immunol. 111 (2015), https://doi.org/10.1002/0471142735.ima03bs111.
[33] C.-H. Liu, S.-W. Kuo, L.-M. Hsu, S.-C. Huang, C.-H. Wang, P.-R. Tsai, Y.-S. Chen, T.-S. Jou, W.-J. Ko, Peroxiredoxin 1 induces inflammatory cytokine response and predicts outcome of cardiogenic shock patients necessitating extracorporeal membrane oxygenation: an observational cohort study and translational approach, J. Transl. Med. 14 (2016) 114, https://doi.org/10.1186/s12967-016-0869-x.
[34] A. Dallagi, J. Girouard, J. Hamelin-Morrissette, R. Dadzie, L. Laurent, C. Vaillancourt, J. Lafond, C. Carrier, C. Reyes-Moreno, The activating effect of IFN-γ on monocytes/macrophages is regulated by the LIF–trophoblast–IL-10 axis via Stat1 inhibition and Stat3 activation, Cell. Mol. Immunol. 12 (2015) 326–341, https://doi.org/10.1038/cmi.2014.50.
[35] F. Meng, C.A. Lowell, Lipopolysaccharide (LPS)-induced macrophage activation and signal transduction in the absence of Src-family kinases Hck, Fgr, and Lyn, J. Exp. Med. 185 (1997) 1661–1670, https://doi.org/10.1084/jem.185.9.1661.
[36] I. Belhassena, W. Nouari, A. Messaoud, M. Nouar, M. Brahimi, S.-A.C. Lamara, M. Aribi, Aspirin enhances regulatory functional activities of monocytes and downregulates CD16 and CD40 expression in myocardial infarction autoinflammatory disease, Int. Immunopharmacol. 83 (2020) 106349, https://doi.org/10.1016/j.intimp.2020.106349.
[37] K. Van Oostveldt, C. Burvenich, F.M. Da Silva, A.M. Massart-Le¨en, Respiratory burst activity in activated and unstimulated isolated bovine blood neutrophils during experimentally induced Escherichia coli mastitis, J. Dairy Res. 66 (1999) 375–383, https://doi.org/10.1017/s0022029999003623.
[38] J.W. Heinecke, B.M. Shapiro, Respiratory burst oxidase of fertilization, Proc. Natl. Acad. Sci. USA 86 (1989) 1259–1263, https://doi.org/10.1073/pnas.86.4.1259.
[39] X. Li, X. Zhang, L. Song, Y. Li, A. Liu, L. Li, M.D. Ne˘si´c, D. Li, L. Peng, C. Wang, Q. Lin, Nanozyme as tumor energy homeostasis disruptor to augment Cascade catalytic therapy, ACS Nano 18 (2024) 34656–34670, https://doi.org/10.1021/acsnano.4c09982.
[40] T. Az-ma, K. Fujii, O. Yuge, Self-limiting enhancement by nitric oxide of oxygen free radical-induced endothelial cell injury: evidence against the dual action of NO as hydroxyl radical donor/scavenger, Br. J. Pharmacol. 119 (1996) 455–462, https://doi.org/10.1111/j.1476-5381.1996.tb15694.x.
[41] W. Ortmann, A. Such, E. Kolaczkowska, Impact of microparticles released during murine systemic inflammation on macrophage activity and reactive nitrogen species regulation, Immunol. Res. 72 (2024) 299–319, https://doi.org/10.1007/s12026-023-09436-7.
[42] M. Nouar, M. Miliani, I. Belhassena, A. Fatmi, M. Aribi, Sodium selenite modulates global activation of Proinflammatory M1-like macrophages, Necroinflammation and M1-like/M2-like dichotomy at the onset of human type 1 diabetes, Endocr. Metab. Immune Disord. Drug, Targets 23 (2023) 1104–1117, https://doi.org/10.2174/1871530323666230201135916.
[43] R.W. Nims, J.C. Cook, M.C. Krishna, D. Christodoulou, C.M.B. Poore, A.M. Miles, M.B. Grisham, D.A. Wink, Colorimetric Assays for Nitric Oxide and Nitrogen Oxide Species Formed from Nitric Oxide Stock Solutions and Donor Compounds, Methods Enzymol., Elsevier, 1996, pp. 93–105, https://doi.org/10.1016/S0076-6879(96)68012-4.
[44] M. Aribi, Macrophage bactericidal assays, Methods Mol. Biol. Clifton NJ 1784 (2018) 135–149, https://doi.org/10.1007/978-1-4939-7837-3_14.
[45] I. Benghalem, W. Meziane, Z. Hadjidj, L. Ysmail-Dahlouk, A. Belamri, K. Mouhadjer, M. Aribi, High-density lipoprotein immunomodulates the functional activities of macrophage and cytokines produced during ex vivo macrophage-CD4 + T cell crosstalk at the recent-onset human type 1 diabetes, Cytokine 96 (2017) 59–70, https://doi.org/10.1016/j.cyto.2017.03.001.
[46] A.W. Segal, Nitroblue-tetrazolium tests, Lancet Lond. Engl. 2 (1974) 1248–1252, https://doi.org/10.1016/s0140-6736(74)90758-2.
[47] J. Müller, P. Alfo¨ldy, E.-M. Lemmel, Nitroblue-tetrazolium test for the functional evaluation of phagocytic cells: a critical analysis of the methodology, Agents Actions 11 (1981) 384–390, https://doi.org/10.1007/BF01982475.
[48] M. García, I. Martínez-Flo´rez, L. Solano-Gallego, N. García, L. Ordeix, The use of the Nitroblue tetrazolium test in blood granulocytes for discriminating bacterial and non-bacterial neutrophilic dermatitis, Vet. Sci. 11 (2024) 634, https://doi.org/10.3390/vetsci11120634.
[49] R. Bellinati-Pires, D. Waitzberg, M. Salgado, M. Carneiro-Sampaio, Functional alterations of human neutrophils by medium-chain triglyceride emulsions: evaluation of phagocytosis, bacterial killing, and oxidative activity, J. Leukoc. Biol. 53 (1993) 404–410, https://doi.org/10.1002/jlb.53.4.404.
[50] R. Koopman, G. Schaart, M.K. Hesselink, Optimisation of oil red O staining permits combination with immunofluorescence and automated quantification of lipids, Histochem. Cell Biol. 116 (2001) 63–68, https://doi.org/10.1007/s004180100297.
[51] R.V. Farese, T.C. Walther, Lipid droplets finally get a little R-E-S-P-E-C-T, Cell 139 (2009) 855–860, https://doi.org/10.1016/j.cell.2009.11.005.
[52] S. Xu, Y. Huang, Y. Xie, T. Lan, K. Le, J. Chen, S. Chen, S. Gao, X. Xu, X. Shen, H. Huang, P. Liu, Evaluation of foam cell formation in cultured macrophages: an improved method with oil red O staining and DiI-oxLDL uptake, Cytotechnology 62 (2010) 473–481, https://doi.org/10.1007/s10616-010-9290-0.
[53] M. Yorek, X. Jiang, S. Liu, J. Hao, J. Yu, A. Avellino, Z. Liu, M. Curry, H. Keen, J. Shao, A. Kanagasabapathy, M. Kong, Y. Xiong, E.R. Sauter, S.L. Sugg, B. Li, FABP4-Mediated Lipid Accumulation and Lipolysis in Tumor Associated Macrophages Promote Breast cancer Metastasis, 2024, https://doi.org/10.1101/2024.07.02.601733.
[54] T. Exner, C.A. Beretta, Q. Gao, C. Afting, I. Romero-Brey, R. Bartenschlager, L. Fehring, M. Poppelreuther, J. Füllekrug, Lipid droplet quantification based on iterative image processing, J. Lipid Res. 60 (2019) 1333–1344, https://doi.org/10.1194/jlr.D092841.
[55] A. Castoldi, L.B. Monteiro, N. van Teijlingen Bakker, D.E. Sanin, N. Rana, M. Corrado, A.M. Cameron, F. Ha¨ssler, M. Matsushita, G. Caputa, R.I. Klein Geltink, J. Büscher, J. Edwards-Hicks, E.L. Pearce, E.J. Pearce, Triacylglycerol synthesis enhances macrophage inflammatory function, Nat. Commun. 11 (2020) 4107, https://doi.org/10.1038/s41467-020-17881-3.
[56] X.A.M.H. van Dierendonck, F. Vrieling, L. Smeehuijzen, L. Deng, J.P. Boogaard, C.-A. Croes, L. Temmerman, S. Wetzels, E. Biessen, S. Kersten, R. Stienstra, Triglyceride breakdown from lipid droplets regulates the inflammatory response in macrophages, Proc. Natl. Acad. Sci. USA 119 (2022) e2114739119, https://doi.org/10.1073/pnas.2114739119.

[57] S. Takano, H. Kaji, F. Hayashi, K. Higashiguchi, S. Joukei, Y. Kido, J. Takahashi, K. Osawa, A Calculation Model for Serum Ionized Calcium Based on an Equilibrium Equation for Complexation, Anal. Chem. Insights 7 (2012), https://doi.org/10.4137/ACI.S9681. ACI.S9681.
[58] O. Bulut, G. Kilic, P.A. Debisarun, R.J. Ro¨ring, S. Sun, M. Kolkman, E. van Rijssen, J. Ten Oever, H. Koenen, L. Barreiro, J. Domínguez-Andre´s, M.G. Netea, Alendronate modulates cytokine responses in healthy young individuals after BCG vaccination, Immunol. Lett. 267 (2024) 106851, https://doi.org/10.1016/j.imlet.2024.106851.
[59] S. Bekkering, R.J.W. Arts, B. Novakovic, I. Kourtzelis, C.D.C.C. van der Heijden, Y. Li, C.D. Popa, R. Ter Horst, J. van Tuijl, R.T. Netea-Maier, F.L. van de Veerdonk, T. Chavakis, L.A.B. Joosten, J.W.M. van der Meer, H. Stunnenberg, N. P. Riksen, M.G. Netea, Metabolic induction of trained immunity through the mevalonate pathway, Cell 172 (2018) 135–146.e9, https://doi.org/10.1016/j.cell.2017.11.025.
[60] H. Nah, J. Yim, S.-G. Lee, J.-B. Lim, J.-H. Kim, Ascorbate oxidase minimizes interference by high-concentration ascorbic acid in Total cholesterol assays, Ann. Lab. Med. 36 (2016) 188–190, https://doi.org/10.3343/alm.2016.36.2.188.
[61] P. Duewell, H. Kono, K.J. Rayner, C.M. Sirois, G. Vladimer, F.G. Bauernfeind, G. S. Abela, L. Franchi, G. Nun˜ez, M. Schnurr, T. Espevik, E. Lien, K.A. Fitzgerald, K. L. Rock, K.J. Moore, S.D. Wright, V. Hornung, E. Latz, NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals, Nature 464 (2010) 1357–1361, https://doi.org/10.1038/nature08938.
[62] Y. Wu, D. Huang, X. Wang, C. Pei, W. Xiao, F. Wang, Z. Wang, Suppression of NLRP3 inflammasome by Platycodin D via the TLR4/MyD88/NF-κB pathway contributes to attenuation of lipopolysaccharide induced acute lung injury in rats, Int. Immunopharmacol. 96 (2021) 107621, https://doi.org/10.1016/j.intimp.2021.107621.
[63] L. Kamentsky, T.R. Jones, A. Fraser, M.-A. Bray, D.J. Logan, K.L. Madden, V. Ljosa, C. Rueden, K.W. Eliceiri, A.E. Carpenter, Improved structure, function and compatibility for CellProfiler: modular high-throughput image analysis software, Bioinforma. Oxf. Engl. 27 (2011) 1179–1180, https://doi.org/10.1093/bioinformatics/btr095.
[64] M. Jackson, A. Krasnodembskaya, Analysis of mitochondrial transfer in direct co-cultures of human monocyte-derived macrophages (MDM) and mesenchymal stem cells (MSC), BIO-Protoc. 7 (2017), https://doi.org/10.21769/BioProtoc.2255.
[65] D.A. Rodeberg, M.S. Chaet, R.C. Bass, M.S. Arkovitz, V.F. Garcia, Nitric oxide: an overview, Am. J. Surg. 170 (1995) 292–303, https://doi.org/10.1016/s0002-9610(05)80017-0.
[66] N. Yagihashi, H. Kasajima, S. Sugai, K. Matsumoto, Y. Ebina, T. Morita, T. Murakami, S. Yagihashi, Increased in situ expression of nitric oxide synthase in human colorectal cancer, Virchows Arch. 436 (2000) 109–114, https://doi.org/10.1007/PL00008208.
[67] A. Siegert, C. Rosenberg, W.D. Schmitt, C. Denkert, S. Hauptmann, Nitric oxide of human colorectal adenocarcinoma cell lines promotes tumour cell invasion, Br. J. Cancer 86 (2002) 1310–1315, https://doi.org/10.1038/sj.bjc.6600224.
[68] C. Wittmann, P. Chockley, S.K. Singh, L. Pase, G.J. Lieschke, C. Grabher, Hydrogen peroxide in inflammation: messenger, guide, and assassin, Adv. Hematol. 2012 (2012) 1–6, https://doi.org/10.1155/2012/541471.
[69] R. Colavitti, G. Pani, B. Bedogni, R. Anzevino, S. Borrello, J. Waltenberger, T. Galeotti, Reactive oxygen species as downstream mediators of Angiogenic signaling by vascular endothelial growth factor Receptor-2/KDR, J. Biol. Chem. 277 (2002) 3101–3108, https://doi.org/10.1074/jbc.M107711200.
[70] S. Miyamoto, T. Narita, M. Komiya, G. Fujii, T. Hamoya, R. Nakanishi, S. Tamura, Y. Kurokawa, M. Takahashi, M. Mutoh, Novel screening system revealed that intracellular cholesterol trafficking can be a good target for colon cancer prevention, Sci. Rep. 9 (2019) 6192, https://doi.org/10.1038/s41598-019-42363-y.
[71] S. Lin, Y. Li, A.A. Zamyatnin, J. Werner, A.V. Bazhin, Reactive oxygen species and colorectal cancer, J. Cell. Physiol. 233 (2018) 5119–5132, https://doi.org/10.1002/jcp.26356.
[72] W. Meziane, Z. Mekkaoui, I. Hai, K. Kacimi, K. Djilali, C. Touil-Boukoffa, G. Lefranc, A. Fernandez, N. Lamb, F. Mennechet, M. Aribi, Combination of metformin with sodium selenite induces a functional phenotypic switch of human GM-CSF monocyte-derived macrophages, Int. Immunopharmacol. 73 (2019) 212–224, https://doi.org/10.1016/j.intimp.2019.05.004.
[73] R.M. Nassif, E. Chalhoub, P. Chedid, M. Hurtado-Nedelec, E. Raya, P.M.-C. Dang, J.-C. Marie, J. El-Benna, Metformin inhibits ROS production by human M2 macrophages via the activation of AMPK, Biomedicines 10 (2022) 319, https://doi.org/10.3390/biomedicines10020319.
[74] Ł. Bułdak, K. Łabuzek, R.J. Bułdak, M. Kozłowski, G. Machnik, S. Liber, D. Suchy, A. Duława-Bułdak, B. Okopien´, Metformin affects macrophages' phenotype and improves the activity of glutathione peroxidase, superoxide dismutase, catalase and decreases malondialdehyde concentration in a partially AMPK-independent manner in LPS-stimulated human monocytes/macrophages, Pharmacol. Rep. 66 (2014) 418–429, https://doi.org/10.1016/j.pharep.2013.11.008.
[75] F. Cianchi, C. Cortesini, O. Fantappie`, L. Messerini, N. Schiavone, A. Vannacci, S. Nistri, I. Sardi, G. Baroni, C. Marzocca, F. Perna, R. Mazzanti, P. Bechi, E. Masini, Inducible nitric oxide synthase expression in human colorectal cancer: correlation with tumor angiogenesis, Am. J. Pathol. 162 (2003) 793–801, https://doi.org/10.1016/S0002-9440(10)63876-X.
[76] M.P. Lisanti, U.E. Martinez-Outschoorn, Z. Lin, S. Pavlides, D. Whitaker-Menezes, R.G. Pestell, A. Howell, F. Sotgia, Hydrogen peroxide fuels aging, inflammation, cancer metabolism and metastasis: the seed and soil also needs "fertilizer,", Cell Cycle Georget. Tex 10 (2011) 2440–2449, https://doi.org/10.4161/cc.10.15.16870.
[77] U.E. Martinez-Outschoorn, Z. Lin, C. Trimmer, N. Flomenberg, C. Wang, S. Pavlides, R.G. Pestell, A. Howell, F. Sotgia, M.P. Lisanti, Cancer cells metabolically "fertilize" the tumor microenvironment with hydrogen peroxide, driving the Warburg effect: implications for PET imaging of human tumors, Cell Cycle Georget. Tex 10 (2011) 2504–2520, https://doi.org/10.4161/cc.10.15.16585.
[78] G.E. Villalpando-Rodriguez, S.B. Gibson, Reactive oxygen species (ROS) regulates different types of cell death by acting as a rheostat, Oxidative Med. Cell. Longev. 2021 (2021) 9912436, https://doi.org/10.1155/2021/9912436.
[79] V. Nogueira, N. Hay, Molecular pathways: reactive oxygen species homeostasis in cancer cells and implications for cancer therapy, Clin. Cancer res, Off. J. Am. Assoc. Cancer Res. 19 (2013) 4309–4314, https://doi.org/10.1158/1078-0432.CCR-12-1424.
[80] S. Li, H. Han, K. Yang, X. Li, L. Ma, Z. Yang, Y.-X. Zhao, Emerging role of metabolic reprogramming in the immune microenvironment and immunotherapy of thyroid cancer, Int. Immunopharmacol. 144 (2025) 113702, https://doi.org/10.1016/j.intimp.2024.113702.
[81] Y.-J. Xu, R.-S. Wei, X.-H. Li, Q. Li, J.-R. Yu, X.-F. Zhuang, MiR-421 promotes lipid metabolism by targeting PTEN via activating PI3K/AKT/mTOR pathway in non-small cell lung cancer, Epigenomics (2022), https://doi.org/10.2217/epi-2021-0229.
[82] B. Han, X. Lin, H. Hu, Regulation of PI3K signaling in cancer metabolism and PI3K-targeting therapy, Transl. Breast Cancer res. J. Focus. Transl. Res, Breast Cancer 5 (2024) 33, https://doi.org/10.21037/tbcr-24-29.
[83] X. Wu, G. Daniels, P. Lee, M.E. Monaco, Lipid metabolism in prostate cancer, Am. J. Clin. Exp. Urol. 2 (2014) 111–120.
[84] H.-R. Jin, J. Wang, Z.-J. Wang, M.-J. Xi, B.-H. Xia, K. Deng, J.-L. Yang, Lipid metabolic reprogramming in tumor microenvironment: from mechanisms to therapeutics, J. Hematol. Oncol, J. Hematol. Oncol. 16 (2023) 103, https://doi.org/10.1186/s13045-023-01498-2.
[85] F.A. Lagunas-Rangel, Cholesterol effects on the tumor immune microenvironment: from fundamental concepts to mechanisms and implications, Front. Oncol. 15 (2025) 1579054, https://doi.org/10.3389/fonc.2025.1579054.
[86] Y. Fu, T. Zou, X. Shen, P.J. Nelson, J. Li, C. Wu, J. Yang, Y. Zheng, C. Bruns, Y. Zhao, L. Qin, Q. Dong, Lipid metabolism in cancer progression and therapeutic strategies, MedComm 2 (2021) 27–59, https://doi.org/10.1002/mco2.27.
[87] S. Zhang, K. Lv, Z. Liu, R. Zhao, F. Li, Fatty acid metabolism of immune cells: a new target of tumour immunotherapy, Cell Death Dis. 10 (2024) 39, https://doi.org/10.1038/s41420-024-01807-9.
[88] G. Zhou, R. Myers, Y. Li, Y. Chen, X. Shen, J. Fenyk-Melody, M. Wu, J. Ventre, T. Doebber, N. Fujii, N. Musi, M.F. Hirshman, L.J. Goodyear, D.E. Moller, Role of AMP-activated protein kinase in mechanism of metformin action, J. Clin. Invest. 108 (2001) 1167–1174, https://doi.org/10.1172/JCI13505.
[89] G.R. Steinberg, D. Carling, AMP-activated protein kinase: the current landscape for drug development, Nat. Rev. Drug Discov. 18 (2019) 527–551, https://doi.org/10.1038/s41573-019-0019-2.
[90] Y. Li, S. Xu, M.M. Mihaylova, B. Zheng, X. Hou, B. Jiang, O. Park, Z. Luo, E. Lefai, J.Y.-J. Shyy, B. Gao, M. Wierzbicki, T.J. Verbeuren, R.J. Shaw, R.A. Cohen, M. Zang, AMPK phosphorylates and inhibits SREBP activity to attenuate hepatic steatosis and atherosclerosis in diet-induced insulin-resistant mice, Cell Metab. 13 (2011) 376–388, https://doi.org/10.1016/j.cmet.2011.03.009.
[91] W.J. Lee, M. Kim, H.-S. Park, H.S. Kim, M.J. Jeon, K.S. Oh, E.H. Koh, J.C. Won, M.-S. Kim, G.T. Oh, M. Yoon, K.-U. Lee, J.-Y. Park, AMPK activation increases fatty acid oxidation in skeletal muscle by activating PPARalpha and PGC-1, Biochem. Biophys. Res. Commun. 340 (2006) 291–295, https://doi.org/10.1016/j.bbrc.2005.12.011.
[92] Z.-Q. Lan, Z.-Y. Ge, S.-K. Lv, B. Zhao, C.-X. Li, The regulatory role of lipophagy in central nervous system diseases, Cell Death Dis. 9 (2023) 229, https://doi.org/10.1038/s41420-023-01504-z.
[93] S. Wang, H. Li, M. Yuan, H. Fan, Z. Cai, Role of AMPK in autophagy, Front. Physiol. 13 (2022) 1015500, https://doi.org/10.3389/fphys.2022.1015500.
[94] Z.-L. Qin, Q.-F. Yao, P. Zhao, H. Ren, Z.-T. Qi, Zika virus infection triggers lipophagy by stimulating the AMPK-ULK1 signaling in human hepatoma cells, Front. Cell. Infect. Microbiol. 12 (2022) 959029, https://doi.org/10.3389/fcimb.2022.959029.
[95] W. Zhang, L. Xu, L. Zhu, Y. Liu, S. Yang, M. Zhao, Lipid droplets, the central hub integrating cell metabolism and the immune system, Front. Physiol. 12 (2021) 746749, https://doi.org/10.3389/fphys.2021.746749.
[96] F.S. Pereira-Dutra, P.T. Bozza, Lipid droplets diversity and functions in inflammation and immune response, Expert Rev. Proteomics 18 (2021) 809–825, https://doi.org/10.1080/14789450.2021.1995356.
[97] M. Lee-Rueckert, J. Lappalainen, P.T. Kovanen, J.C. Escola-Gil, Lipid-laden macrophages and inflammation in atherosclerosis and Cancer: an integrative view, Front. Cardiovasc. Med. 9 (2022) 777822, https://doi.org/10.3389/fcvm.2022.777822.
[98] R.J.W. Arts, B. Novakovic, R. Ter Horst, A. Carvalho, S. Bekkering, E. Lachmandas, F. Rodrigues, R. Silvestre, S.-C. Cheng, S.-Y. Wang, E. Habibi, L. G. Gonçalves, I. Mesquita, C. Cunha, A. van Laarhoven, F.L. van de Veerdonk, D. L. Williams, J.W.M. van der Meer, C. Logie, L.A. O'Neill, C.A. Dinarello, N. P. Riksen, R. van Crevel, C. Clish, R.A. Notebaart, L.A.B. Joosten, H. G. Stunnenberg, R.J. Xavier, M.G. Netea, Glutaminolysis and fumarate accumulation integrate Immunometabolic and epigenetic programs in trained immunity, Cell Metab. 24 (2016) 807–819, https://doi.org/10.1016/j.cmet.2016.10.008.

[99] H. Liu, Y. Liu, Y. Zhou, X. Chen, S. Pan, Q. Zhou, H. Ji, X. Zhu, TM7SF2-induced lipid reprogramming promotes cell proliferation and migration via CPT1A/Wnt/ β-catenin axis in cervical cancer cells, Cell Death Dis. 10 (2024) 207, https://doi.org/10.1038/s41420-024-01975-8.
[100] H. Tanton, T. Sewastianik, H.-S. Seo, D. Remillard, R.S. Pierre, P. Bala, D. Aitymbayev, P. Dennis, K. Adler, E. Geffken, Z. Yeoh, N. Vangos, F. Garbicz, D. Scott, N. Sethi, J. Bradner, S. Dhe-Paganon, R.D. Carrasco, A novel β-catenin/BCL9 complex inhibitor blocks oncogenic Wnt signaling and disrupts cholesterol homeostasis in colorectal cancer, Sci. Adv. 8 (2022) eabm3108, https://doi.org/10.1126/sciadv.abm3108.
[101] D. Wang, R.N. Dubois, Prostaglandins and cancer, Gut 55 (2006) 115–122, https://doi.org/10.1136/gut.2004.047100.
[102] P. Ekambaram, W. Lambiv, R. Cazzolli, A.W. Ashton, K.V. Honn, The thromboxane synthase and receptor signaling pathway in cancer: an emerging paradigm in cancer progression and metastasis, Cancer Metastasis Rev. 30 (2011) 397–408, https://doi.org/10.1007/s10555-011-9297-9.
[103] D. Wang, R.N. Dubois, Eicosanoids and cancer, Nat. Rev. Cancer 10 (2010) 181–193, https://doi.org/10.1038/nrc2809.
[104] Y. Jin, Y. Tan, J. Wu, Z. Ren, Lipid droplets: a cellular organelle vital in cancer cells, Cell Death Dis. 9 (2023) 254, https://doi.org/10.1038/s41420-023-01493-z.
[105] S. Ugel, S. Can`e, F. De Sanctis, V. Bronte, Monocytes in the tumor microenvironment, Annu. Rev. Pathol. Mech. Dis. 16 (2021) 93–122, https://doi.org/10.1146/annurev-pathmechdis-012418-013058.
[106] S. Wang, W. Yan, L. Kong, S. Zuo, J. Wu, C. Zhu, H. Huang, B. He, J. Dong, J. Wei, Oncolytic viruses engineered to enforce cholesterol efflux restore tumor-associated macrophage phagocytosis and anti-tumor immunity in glioblastoma, Nat. Commun. 14 (2023) 4367, https://doi.org/10.1038/s41467-023-39683-z.
[107] X. Zhu, H. Zhang, W. Peng, S. Gao, Z. Yang, J. Zhang, X. Liu, Autophagy impairment is involved in midazolam-induced lipid droplet accumulation and consequent phagocytosis decrease in BV2 cells, Biochem. Biophys. Res. Commun. 643 (2023) 147–156, https://doi.org/10.1016/j.bbrc.2022.12.067.
[108] Y. Jia, M. Guo, Epigenetic changes in colorectal cancer, Chin. J. Cancer 32 (2013) 21–30, https://doi.org/10.5732/cjc.011.10245.
[109] Z. Qian, Q. Shen, X. Yang, Y. Qiu, W. Zhang, The role of extracellular vesicles: an epigenetic view of the Cancer microenvironment, Biomed. Res. Int. 2015 (2015) 649161, https://doi.org/10.1155/2015/649161.
[110] A. Roquilly, C. Jacqueline, M. Davieau, A. Molle´, A. Sadek, C. Fourgeux, P. Rooze, A. Broquet, B. Misme-Aucouturier, T. Chaumette, M. Vourc'h, R. Cinotti, N. Marec, V. Gauttier, H.E.G. McWilliam, F. Altare, J. Poschmann, J. A. Villadangos, K. Asehnoune, Alveolar macrophages are epigenetically altered after inflammation, leading to long-term lung immunoparalysis, Nat. Immunol. 21 (2020) 636–648, https://doi.org/10.1038/s41590-020-0673-x.
[111] P. Rougerie, V. Miskolci, D. Cox, Generation of membrane structures during phagocytosis and chemotaxis of macrophages: role and regulation of the actin cytoskeleton, Immunol. Rev. 256 (2013) 222–239, https://doi.org/10.1111/imr.12118.
[112] L. Campana, P.J. Starkey Lewis, A. Pellicoro, R.L. Aucott, J. Man, E. O'Duibhir, S. E. Mok, S. Ferreira-Gonzalez, E. Livingstone, S.N. Greenhalgh, K.L. Hull, T. J. Kendall, D. Vernimmen, N.C. Henderson, L. Boulter, C.D. Gregory, Y. Feng, S. M. Anderton, S.J. Forbes, J.P. Iredale, The STAT3-IL-10-IL-6 pathway is a novel regulator of macrophage Efferocytosis and phenotypic conversion in sterile liver injury, J. Immunol. Baltim. Md 1950 (200) (2018) 1169–1187, https://doi.org/10.4049/jimmunol.1701247.
[113] A. Ge, S. Wang, B. Miao, M. Yan, Effects of metformin on the expression of AMPK and STAT3 in the spinal dorsal horn of rats with neuropathic pain, Mol. Med. Rep. 17 (2018) 5229–5237, https://doi.org/10.3892/mmr.2018.8541.
[114] X. Wang, X. Luo, C. Chen, Y. Tang, L. Li, B. Mo, H. Liang, S. Yu, The Ap-2α/Elk-1 axis regulates Sirpα-dependent tumor phagocytosis by tumor-associated macrophages in colorectal cancer, Signal Transduct. Target. Ther. 5 (2020) 35, https://doi.org/10.1038/s41392-020-0124-z.
[115] A.J. Freemerman, A.R. Johnson, G.N. Sacks, J.J. Milner, E.L. Kirk, M.A. Troester, A.N. Macintyre, P. Goraksha-Hicks, J.C. Rathmell, L. Makowski, Metabolic reprogramming of macrophages: glucose transporter 1 (GLUT1)-mediated glucose metabolism drives a proinflammatory phenotype, J. Biol. Chem. 289 (2014) 7884–7896, https://doi.org/10.1074/jbc.M113.522037.
[116] M.J. Cantoria, L.G. Boros, E.J. Meuillet, Contextual inhibition of fatty acid synthesis by metformin involves glucose-derived acetyl-CoA and cholesterol in pancreatic tumor cells, Metabolomics Off. J. Metabolomic Soc. 10 (2014) 91–104, https://doi.org/10.1007/s11306-013-0555-4.
[117] N. Duan, Y. Zhang, S. Tan, J. Sun, M. Ye, H. Gao, K. Pu, M. Wu, Q. Wang, Q. Zhai, Therapeutic targeting of STING-TBK1-IRF3 signalling ameliorates chronic stress induced depression-like behaviours by modulating neuroinflammation and microglia phagocytosis, Neurobiol. Dis. 169 (2022) 105739, https://doi.org/10.1016/j.nbd.2022.105739.
[118] S. Shin, B. Hyun, A. Lee, H. Kong, S. Han, C.-K. Lee, N.-J. Ha, K. Kim, Metformin suppresses MHC-restricted antigen presentation by inhibiting co-stimulatory factors and MHC molecules in APCs, Biomol. Ther. 21 (2013) 35–41, https://doi.org/10.4062/biomolther.2012.094.
[119] M.A. Gronski, J.M. Kinchen, I.J. Juncadella, N.C. Franc, K.S. Ravichandran, An essential role for calcium flux in phagocytes for apoptotic cell engulfment and the anti-inflammatory response, Cell Death Differ. 16 (2009) 1323–1331, https://doi.org/10.1038/cdd.2009.55.
[120] S. Mylvaganam, S.A. Freeman, S. Grinstein, The cytoskeleton in phagocytosis and macropinocytosis, Curr. Biol. CB 31 (2021) R619–R632, https://doi.org/10.1016/j.cub.2021.01.036.
[121] P. Nunes, N. Demaurex, The role of calcium signaling in phagocytosis, J. Leukoc. Biol. 88 (2010) 57–68, https://doi.org/10.1189/jlb.0110028.
[122] S.R. Barger, N.C. Gauthier, M. Krendel, Squeezing in a meal: myosin functions in phagocytosis, Trends Cell Biol. 30 (2020) 157–167, https://doi.org/10.1016/j.tcb.2019.11.002.
[123] M.G. Dalghi, M. Ferreira-Gomes, J.P. Rossi, Regulation of the plasma membrane calcium ATPases by the actin cytoskeleton, Biochem. Biophys. Res. Commun. 506 (2018) 347–354, https://doi.org/10.1016/j.bbrc.2017.11.151.
[124] V. Gerke, F.N.E. Gavins, M. Geisow, T. Grewal, J.K. Jaiswal, J. Nylandsted, U. Rescher, Annexins-a family of proteins with distinctive tastes for cell signaling and membrane dynamics, Nat. Commun. 15 (2024) 1574, https://doi.org/10.1038/s41467-024-45954-0.
[125] Q. Zheng, H. Zhang, H. Zhao, Y. Chen, H. Yang, T. Li, Q. Cai, Y. Chen, Y. Wang, M. Zhang, H. Zhang, Ca2+/calmodulin-dependent protein kinase II β decodes ER Ca2+ transients to trigger autophagosome formation, Mol. Cell 85 (2025) 620–637.e6, https://doi.org/10.1016/j.molcel.2024.12.005.
[126] S. Dewitt, M.B. Hallett, Calpain activation by Ca2+ and its role in phagocytosis, Adv. Exp. Med. Biol. 1246 (2020) 129–151, https://doi.org/10.1007/978-3-030-40406-2_8.
[127] Y.C. Eskiocak, Z.O. Ayyildiz, S. Gunalp, A. Korkmaz, D.G. Helvaci, Y. Dogan, D. Sag, G. Wingender, The Ca2+ concentration impacts the cytokine production of mouse and human lymphoid cells and the polarization of human macrophages in vitro, PLoS One 18 (2023) e0282037, https://doi.org/10.1371/journal.pone.0282037.
[128] A. Rao, P.G. Hogan, Calcium signaling in cells of the immune and hematopoietic systems, Immunol. Rev. 231 (2009) 5–9, https://doi.org/10.1111/j.1600-065X.2009.00823.x.
[129] V. Rogovskii, Tumor-produced immune regulatory factors as a therapeutic target in cancer treatment, Front. Immunol. 15 (2024) 1416458, https://doi.org/10.3389/fimmu.2024.1416458.
[130] J. Zong, A.A. Keskinov, G.V. Shurin, M.R. Shurin, Tumor-derived factors modulating dendritic cell function, Cancer Immunol. Immunother. CII 65 (2016) 821–833, https://doi.org/10.1007/s00262-016-1820-y.
[131] S.M. Gilbert, C.J. Oliphant, S. Hassan, A.L. Peille, P. Bronsert, S. Falzoni, F. Di Virgilio, S. McNulty, R. Lara, ATP in the tumour microenvironment drives expression of nfP2X7, a key mediator of cancer cell survival, Oncogene 38 (2019) 194–208, https://doi.org/10.1038/s41388-018-0426-6.
[132] J. Canton, D. Schlam, C. Breuer, M. Gütschow, M. Glogauer, S. Grinstein, Calcium-sensing receptors signal constitutive macropinocytosis and facilitate the uptake of NOD2 ligands in macrophages, Nat. Commun. 7 (2016) 11284, https://doi.org/10.1038/ncomms11284.
[133] G.-Y. Jang, J.W. Lee, Y.S. Kim, S.E. Lee, H.D. Han, K.-J. Hong, T.H. Kang, Y.-M. Park, Interactions between tumor-derived proteins and toll-like receptors, Exp. Mol. Med. 52 (2020) 1926–1935, https://doi.org/10.1038/s12276-020-00540-4.
[134] F. Podo, L. Paris, S. Cecchetti, F. Spadaro, L. Abalsamo, C. Ramoni, A. Ricci, M. E. Pisanu, F. Sardanelli, R. Canese, E. Iorio, Activation of phosphatidylcholine-specific phospholipase C in breast and ovarian Cancer: impact on MRS-detected choline metabolic profile and perspectives for targeted therapy, Front. Oncol. 6 (2016) 171, https://doi.org/10.3389/fonc.2016.00171.
[135] M.J. Berridge, The inositol trisphosphate/calcium signaling pathway in health and disease, Physiol. Rev. 96 (2016) 1261–1296, https://doi.org/10.1152/physrev.00006.2016.
[136] M.Y. Sun, M. Geyer, Y.A. Komarova, IP3 receptor signaling and endothelial barrier function, Cell. Mol. Life Sci. CMLS 74 (2017) 4189–4207, https://doi.org/10.1007/s00018-017-2624-8.
[137] V. Nascimento Da Conceicao, Y. Sun, K. Ramachandran, A. Chauhan, A. Raveendran, M. Venkatesan, B. DeKumar, S. Maity, N. Vishnu, G.A. Kotsakis, P. F. Worley, D.L. Gill, B.B. Mishra, M. Madesh, B.B. Singh, Resolving macrophage polarization through distinct Ca2+ entry channel that maintains intracellular signaling and mitochondrial bioenergetics, iScience 24 (2021) 103339, https://doi.org/10.1016/j.isci.2021.103339.
[138] X. Zheng, K. Turkowski, J. Mora, B. Brüne, W. Seeger, A. Weigert, R. Savai, Redirecting tumor-associated macrophages to become tumoricidal effectors as a novel strategy for cancer therapy, Oncotarget 8 (2017) 48436–48452, https://doi.org/10.18632/oncotarget.17061.
[139] T. Kwak, F. Wang, H. Deng, T. Condamine, V. Kumar, M. Perego, A. Kossenkov, L. J. Montaner, X. Xu, W. Xu, C. Zheng, L.M. Schuchter, R.K. Amaravadi, T. C. Mitchell, G.C. Karakousis, C. Mulligan, B. Nam, G. Masters, N. Hockstein, J. Bennett, Y. Nefedova, D.I. Gabrilovich, Distinct populations of immune-suppressive macrophages differentiate from Monocytic myeloid-derived suppressor cells in Cancer, Cell Rep. 33 (2020) 108571, https://doi.org/10.1016/j.celrep.2020.108571.
[140] T. Bose, A. Cie´slar-Pobuda, E. Wiechec, Role of ion channels in regulating Ca^{2+} homeostasis during the interplay between immune and cancer cells, Cell Death Dis. 6 (2015) e1648, https://doi.org/10.1038/cddis.2015.23.
[141] R. Huang, T. Kang, S. Chen, The role of tumor-associated macrophages in tumor immune evasion, J. Cancer Res. Clin. Oncol. 150 (2024) 238, https://doi.org/10.1007/s00432-024-05777-4.
[142] M. Vig, J.-P. Kinet, Calcium signaling in immune cells, Nat. Immunol. 10 (2009) 21–27, https://doi.org/10.1038/ni.f.220.
[143] S. Zheng, X. Wang, D. Zhao, H. Liu, Y. Hu, Calcium homeostasis and cancer: insights from endoplasmic reticulum-centered organelle communications, Trends Cell Biol. 33 (2023) 312–323, https://doi.org/10.1016/j.tcb.2022.07.004.
[144] E. Fontaine, Metformin-induced mitochondrial complex I inhibition: facts, uncertainties, and consequences, Front. Endocrinol. 9 (2018) 753, https://doi.org/10.3389/fendo.2018.00753.

[145] J. Feng, X. Wang, X. Ye, I. Ares, B. Lopez-Torres, M. Martínez, M.-R. Martínez-Larran˜aga, X. Wang, A. Anado´n, M.-A. Martínez, Mitochondria as an important target of metformin: the mechanism of action, toxic and side effects, and new therapeutic applications, Pharmacol. Res. 177 (2022) 106114, https://doi.org/10.1016/j.phrs.2022.106114.

[146] A.S. Chauhan, X. Liu, J. Jing, H. Lee, R.K. Yadav, J. Liu, Y. Zhou, B. Gan, STIM2 interacts with AMPK and regulates calcium-induced AMPK activation, FASEB, J. Off. Publ. Fed. Am. Soc. Exp. Biol. 33 (2019) 2957–2970, https://doi.org/10.1096/fj.201801225R.

[147] F. Lang, M. Fo¨ller, Regulation of ion channels and transporters by AMP-activated kinase (AMPK), Channels Austin Tex 8 (2014) 20–28, https://doi.org/10.4161/chan.27423.

[148] Y. Xia, S. Shen, I.M. Verma, NF-κB, an active player in human cancers, Cancer, Immunol. Res. 2 (2014) 823–830, https://doi.org/10.1158/2326-6066.CIR-14-0112.

[149] M. Mussbacher, M. Derler, J. Basílio, J.A. Schmid, NF-κB in monocytes and macrophages - an inflammatory master regulator in multitalented immune cells, Front. Immunol. 14 (2023) 1134661, https://doi.org/10.3389/fimmu.2023.1134661.

[150] Y. Marandi, S. Hashemzade, H. Tayebinia, J. Karimi, A. Zamani, I. Khodadadi, NLRP3-inflammasome activation is associated with epithelial-mesenchymal transition and progression of colorectal cancer, Iran. J. Basic Med. Sci. 24 (2021) 483–492, https://doi.org/10.22038/ijbms.2021.52355.11835.

[151] C.S. Mantsounga, C. Lee, J. Neverson, S. Sharma, A. Healy, J.M. Berus, C. Parry, N.M. Ceneri, F. Lo´pez-Gira´ldez, H.J. Chun, Q. Lu, F. Sellke, G. Choudhary, A. R. Morrison, Macrophage IL-1β promotes arteriogenesis by autocrine STAT3- and NF-κB-mediated transcription of pro-angiogenic VEGF-A, Cell Rep. 38 (2022) 110309, https://doi.org/10.1016/j.celrep.2022.110309.

[152] H. Xian, Y. Liu, A. Rundberg Nilsson, R. Gatchalian, T.R. Crother, W. G. Tourtellotte, Y. Zhang, G.R. Aleman-Muench, G. Lewis, W. Chen, S. Kang, M. Luevanos, D. Trudler, S.A. Lipton, P. Soroosh, J. Teijaro, J.C. de la Torre, M. Arditi, M. Karin, E. Sanchez-Lopez, Metformin inhibition of mitochondrial ATP and DNA synthesis abrogates NLRP3 inflammasome activation and pulmonary inflammation, Immunity 54 (2021) 1463–1477.e11, https://doi.org/10.1016/j.immuni.2021.05.004.

[153] R.-W. Du, W.-G. Bu, Metformin improves depressive-like symptoms in mice via inhibition of peripheral and central NF-κB-NLRP3 inflammation activation, Exp. Brain Res. 238 (2020) 2549–2556, https://doi.org/10.1007/s00221-020-05911-x.

[154] Y. Hattori, K. Suzuki, S. Hattori, K. Kasai, Metformin inhibits cytokine-induced nuclear factor kappaB activation via AMP-activated protein kinase activation in vascular endothelial cells, Hypertens. Dallas Tex 1979 (47) (2006) 1183–1188, https://doi.org/10.1161/01.HYP.0000221429.94591.72.

[155] S.-M. Ong, E. Hadadi, T.-M. Dang, W.-H. Yeap, C.T.-Y. Tan, T.-P. Ng, A. Larbi, S.-C. Wong, The pro-inflammatory phenotype of the human non-classical monocyte subset is attributed to senescence, Cell Death Dis. 9 (2018) 266, https://doi.org/10.1038/s41419-018-0327-1.

[156] R. Mukherjee, P. Kanti Barman, P. Kumar Thatoi, R. Tripathy, B. Kumar Das, B. Ravindran, Non-classical monocytes display inflammatory features: validation in Sepsis and systemic lupus erythematous, Sci. Rep. 5 (2015) 13886, https://doi.org/10.1038/srep13886.

[157] C.-F. Chiang, T.-T. Chao, Y.-F. Su, C.-C. Hsu, C.-Y. Chien, K.-C. Chiu, S.-G. Shiah, C.-H. Lee, S.-Y. Liu, Y.-S. Shieh, Metformin-treated cancer cells modulate macrophage polarization through AMPK-NF-κB signaling, Oncotarget 8 (2017) 20706–20718, https://doi.org/10.18632/oncotarget.14982.

[158] H. Lin, H. Ao, G. Guo, M. Liu, The role and mechanism of metformin in inflammatory diseases, J. Inflamm. Res. 16 (2023) 5545–5564, https://doi.org/10.2147/JIR.S436147.

[159] A. Saito, J. Kitayama, H. Horie, K. Koinuma, H. Ohzawa, H. Yamaguchi, H. Kawahira, T. Mimura, A.K. Lefor, N. Sata, Metformin changes the immune microenvironment of colorectal cancer in patients with type 2 diabetes mellitus, Cancer Sci. 111 (2020) 4012–4020, https://doi.org/10.1111/cas.14615.

[160] J. Liu, X. Li, Y. Li, Q. Gong, K. Luo, Metformin-based nanomedicines for reprogramming tumor immune microenvironment, Theranostics 15 (2025) 993–1016, https://doi.org/10.7150/thno.104872.

[161] X. Liu, P. Yu, Y. Xu, Y. Wang, J. Chen, F. Tang, Z. Hu, J. Zhou, L. Liu, W. Qiu, Y. Ye, Y. Jia, W. Yao, J. Long, Z. Zeng, Metformin induces tolerogenicity of dendritic cells by promoting metabolic reprogramming, Cell. Mol. Life Sci. CMLS 80 (2023) 283, https://doi.org/10.1007/s00018-023-04932-3.

[162] S. Verdura, E. Cuya`s, B. Martin-Castillo, J.A. Menendez, Metformin as an archetype immuno-metabolic adjuvant for cancer immunotherapy, Oncoimmunology 8 (2019) e1633235, https://doi.org/10.1080/2162402X.2019.1633235.

[163] L. Ding, G. Liang, Z. Yao, J. Zhang, R. Liu, H. Chen, Y. Zhou, H. Wu, B. Yang, Q. He, Metformin prevents cancer metastasis by inhibiting M2-like polarization of tumor associated macrophages, Oncotarget 6 (2015) 36441–36455, https://doi.org/10.18632/oncotarget.5541.

[164] J.-C. Wang, X. Sun, Q. Ma, G.-F. Fu, L.-L. Cong, H. Zhang, D.-F. Fan, J. Feng, S.-Y. Lu, J.-L. Liu, G.-Y. Li, P.-J. Liu, Metformin's antitumour and anti-angiogenic activities are mediated by skewing macrophage polarization, J. Cell. Mol. Med. 22 (2018) 3825–3836, https://doi.org/10.1111/jcmm.13655.

[165] E. Cuya`s, S. Verdura, B. Martin-Castillo, J.A. Menendez, Metformin: targeting the Metabolo-epigenetic link in Cancer biology, Front. Oncol. 10 (2020) 620641, https://doi.org/10.3389/fonc.2020.620641.

[166] Z. Kooshan, L. C´ardenas-Piedra, J. Clements, J. Batra, Glycolysis, the sweet appetite of the tumor microenvironment, Cancer Lett. 600 (2024) 217156, https://doi.org/10.1016/j.canlet.2024.217156.

Chapter 3: Conclusion and future prospects

Conclusion

This work demonstrates that the colorectal tumor microenvironment exerts a profound immunometabolic pressure on monocytes, reshaping their redox balance, ionic homeostasis, lipid metabolism, inflammatory signaling, and phenotypic identity. Exposure of monocytes to tumor-conditioned medium induces a marked oxidative stress characterized by increased production of nitric oxide and hydrogen peroxide, together with a dysregulation of intracellular calcium levels. These early and interconnected alterations act as potent amplifiers of inflammatory signaling and constitute key drivers of monocyte reprogramming within the tumor milieu.

At the metabolic level, our findings reveal that the colorectal tumor microenvironment promotes excessive intracellular cholesterol accumulation, increased triglyceride storage, and enhanced biogenesis of lipid droplets. Far from being passive energy reservoirs, lipid droplets emerge as active metabolic and signaling platforms that contribute to inflammatory amplification. Their accumulation enhances oxidative stress, sustains NF-κB activation, and directly participates in the activation signal of the NLRP3 inflammasome. These lipid-driven mechanisms therefore represent a central axis linking metabolic dysregulation to chronic inflammation in tumor-associated monocytes.

Consistently, activation of the NF-κB/NLRP3 inflammasome axis constitutes a pivotal event in the immunometabolic reprogramming observed in this study. NF-κB-mediated priming leads to increased expression of NLRP3 and pro-IL-1β, while metabolic stressors such as reactive oxygen species, calcium imbalance, cholesterol overload, and lipid droplet accumulation trigger inflammasome activation. The resulting increase in IL-1β production reinforces the inflammatory tumor microenvironment and contributes to the establishment of a self-perpetuating pro-tumoral inflammatory loop.

These molecular and metabolic alterations are accompanied by a marked phenotypic shift in monocytes. Tumor-conditioned medium induces a reduction in CD14 expression and an increase in CD16 expression, consistent with a transition toward a pro-tumoral, inflammation-associated monocyte subset. This phenotypic plasticity highlights the role of monocytes as highly responsive sensors of metabolic and inflammatory cues within the colorectal tumor microenvironment and underscores their contribution to tumor progression through functional and metabolic adaptation.

Within this context, our data identify metformin as a potent immunometabolic modulator capable of counteracting several key alterations induced by the tumor microenvironment. Metformin significantly reduces oxidative stress, restores intracellular calcium homeostasis, limits cholesterol accumulation and lipid droplet formation, and attenuates NF-κB and NLRP3 activation. These combined effects result in decreased IL-1β expression and a partial restoration of a more balanced monocyte phenotype. Importantly, the lack of metformin-mediated recovery of phagocytic activity indicates that its action is selective and context-dependent, suggesting that some tumor-induced functional impairments may be refractory to metabolic modulation alone.

Overall, this work positions metformin as an agent capable of mitigating inflammation and metabolic dysfunction in tumor-associated monocytes rather than as a global functional restorer. It highlights lipid metabolism and inflammasome activation as central nodes in monocyte reprogramming within the colorectal tumor microenvironment and underscores their relevance as therapeutic targets in inflammation-driven cancers.

Perspectives

These findings open several important avenues for future investigation that extend both the mechanistic and translational relevance of this work. First, in vivo validation using preclinical models of colorectal cancer is warranted to confirm the protective immunometabolic effects of metformin within a more complex and physiologically relevant tumor microenvironment. Such models would allow the integration of systemic factors that cannot be fully recapitulated in vitro, including vascularization, immune cell trafficking, stromal interactions, and microbiota-derived signals. In particular, in vivo studies could clarify whether metformin modulates monocyte recruitment, differentiation into tumor-associated macrophages, and functional polarization during tumor progression, thereby validating its role as an immunometabolic regulator at the organismal level.

Second, further investigation into the molecular mechanisms underlying the suppression of lipid accumulation and inflammatory activation is essential. Although AMPK activation is a well-established downstream effect of metformin, its precise contribution to the regulation of cholesterol homeostasis, lipid droplet biogenesis, and inflammasome activity in tumor-exposed monocytes remains to be fully elucidated. Dissecting the involvement of AMPK-dependent and AMPK-independent pathways could reveal novel regulatory nodes linking cellular metabolism to inflammatory signaling. Such insights may identify new therapeutic targets within lipid metabolic pathways, particularly those controlling lipid uptake, esterification, efflux, and storage in immune cells.

Moreover, the potential of metformin as part of combinatorial therapeutic strategies deserves particular attention. Given its capacity to attenuate chronic inflammation and metabolic dysfunction, combining metformin with other immunometabolic modulators or immune checkpoint inhibitors may

result in synergistic reprogramming of the tumor immune microenvironment. By alleviating lipid-driven immunosuppression and inflammasome-mediated inflammation, metformin could enhance the responsiveness of immune cells to immunotherapeutic interventions, thereby improving antitumor efficacy in otherwise resistant tumor settings.

In addition, longitudinal studies examining the impact of metformin on other myeloid populations within the tumor microenvironment would significantly broaden the scope of its immunomodulatory potential. Investigating its effects on myeloid-derived suppressor cells, dendritic cells, and tumor-associated macrophages could provide a more comprehensive understanding of how metformin reshapes myeloid-driven immune regulation over time. Such studies may reveal whether metformin influences the balance between immunosuppressive and immunostimulatory myeloid subsets, contributing to a more favorable antitumoral immune landscape.

Finally, these experimental insights strongly support the need for clinical investigations evaluating the off-label use of metformin in non-diabetic cancer patients. In particular, patients exhibiting high levels of monocyte-driven inflammation, elevated inflammasome activity, or lipid-rich immune phenotypes may represent responsive subpopulations that could benefit from immunometabolic intervention. Stratifying patients based on immunometabolic biomarkers may pave the way toward precision immunometabolic therapy, positioning metformin as a cost-effective and well-tolerated adjuvant in colorectal cancer and potentially other inflammation-associated malignancies.

Chapter 4: Bibliographic references

[1] C.W.-K. Wu, R.N. Lui, Early-onset colorectal cancer: Current insights and future directions, World J. Gastrointest. Oncol. 14 (2022) 230–241. https://doi.org/10.4251/wjgo.v14.i1.230.

[2] A. Hnatyszyn, S. Hryhorowicz, M. Kaczmarek-Ryś, E. Lis, R. Słomski, R.J. Scott, A. Pławski, Colorectal carcinoma in the course of inflammatory bowel diseases, Hered. Cancer Clin. Pract. 17 (2019) 18. https://doi.org/10.1186/s13053-019-0118-4.

[3] Y. Zhang, Y. Zhao, Q. Li, Y. Wang, Macrophages, as a Promising Strategy to Targeted Treatment for Colorectal Cancer Metastasis in Tumor Immune Microenvironment, Front. Immunol. 12 (2021) 685978. https://doi.org/10.3389/fimmu.2021.685978.

[4] J.M. Pitt, A. Marabelle, A. Eggermont, J.-C. Soria, G. Kroemer, L. Zitvogel, Targeting the tumor microenvironment: removing obstruction to anticancer immune responses and immunotherapy, Ann. Oncol. 27 (2016) 1482–1492. https://doi.org/10.1093/annonc/mdw168.

[5] S.I. Grivennikov, F.R. Greten, M. Karin, Immunity, Inflammation, and Cancer, Cell 140 (2010) 883–899. https://doi.org/10.1016/j.cell.2010.01.025.

[6] W. Kuo, J. Chang, C. Chen, N. Tsao, C. Chang, Autophagy drives plasticity and functional polarization of tumor-associated macrophages, IUBMB Life 74 (2022) 157–169. https://doi.org/10.1002/iub.2543.

[7] M. Djaldetti, H. Bessler, Mononuclear cells phagocytic activity affects the crosstalk between immune and cancer cells, Biomed. Pharmacother. 68 (2014) 679–683. https://doi.org/10.1016/j.biopha.2014.08.004.

[8] J. Kim, Y. Yuan, K. Agaronyan, A. Zhao, V.D. Wang, D. Gau, N. Toosi, G. Gupta, H. Essayas, A. Kaminski, J. McGovern, S. Yu, S. Woo, C.J. Lee, S. Gandhi, T. Saber, T. Saleh, B. Hu, Y. Sun, G. Ishikawa, W. Bain, J. Evankovich, L. Chen, H. Yun, E.L. Herzog, C.S. Dela Cruz, C. Ryu, L. Sharma, Damage sensing through TLR9 regulates inflammatory and antiviral responses during influenza infection, Mucosal Immunol. 18 (2025) 537–548. https://doi.org/10.1016/j.mucimm.2025.01.008.

[9] A. Martin, S. Caron, M. Marcotte, P. Bronnec, E. Garneret, N. Martel, G. Maalouf, P. Sève, D. Saadoun, Y. Jamilloux, T. Henry, IFN-γ licenses normal and pathogenic ALPK1/TIFA pathway in human monocytes, iScience 28 (2025) 111563. https://doi.org/10.1016/j.isci.2024.111563.

[10] T.S. Kapellos, L. Bonaguro, I. Gemünd, N. Reusch, A. Saglam, E.R. Hinkley, J.L. Schultze, Human Monocyte Subsets and Phenotypes in Major Chronic Inflammatory

Diseases, Front. Immunol. 10 (2019) 2035. https://doi.org/10.3389/fimmu.2019.02035.

[11] P. Vishnyakova, A. Poltavets, E. Karpulevich, A. Maznina, V. Vtorushina, L. Mikhaleva, E. Kananykhina, A. Lokhonina, S. Kovalchuk, A. Makarov, A. Elchaninov, G. Sukhikh, T. Fatkhudinov, The response of two polar monocyte subsets to inflammation, Biomed. Pharmacother. 139 (2021) 111614. https://doi.org/10.1016/j.biopha.2021.111614.

[12] X. Zhuang, X. Li, J. Zhang, Y. Hu, B. Hu, Y. Shi, Y. Sun, G. Hong, Conditioned medium mimicking the tumor microenvironment augments chemotherapeutic resistance via ataxia-telangiectasia mutated and nuclear factor-κB pathways in gastric cancer cells, Oncol. Rep. (2018). https://doi.org/10.3892/or.2018.6637.

[13] D. Kwart, J. He, S. Srivatsan, C. Lett, J. Golubov, E.M. Oswald, P. Poon, X. Ye, J. Waite, A.G. Zaretsky, S. Haxhinasto, E. Au-Yeung, N.T. Gupta, J. Chiu, C. Adler, S. Cherravuru, E. Malahias, N. Negron, K. Lanza, A. Coppola, M. Ni, H. Song, Y. Wei, G.S. Atwal, L. Macdonald, N.S. Oristian, W. Poueymirou, V. Jankovic, M. Fury, I. Lowy, A.J. Murphy, M.A. Sleeman, B. Wang, D. Skokos, Cancer cell-derived type I interferons instruct tumor monocyte polarization, Cell Rep. 41 (2022)

111769. https://doi.org/10.1016/j.celrep.2022.111769.

[14] T.-Y. Lin, M.-C. Tsai, W. Tu, H.-C. Yeh, S.-C. Wang, S.-P. Huang, C.-Y. Li, Role of the NLRP3 Inflammasome: Insights Into Cancer Hallmarks, Front. Immunol. 11 (2021) 610492. https://doi.org/10.3389/fimmu.2020.610492.

[15] A.L.S. Cruz, E.D.A. Barreto, N.P.B. Fazolini, J.P.B. Viola, P.T. Bozza, Lipid droplets: platforms with multiple functions in cancer hallmarks, Cell Death Dis. 11 (2020) 105. https://doi.org/10.1038/s41419-020-2297-3.

[16] C.-A.W. Ng, A.A. Jiang, E.M.S. Toh, C.H. Ng, Z.H. Ong, S. Peng, H.Y. Tham, R. Sundar, C.S. Chong, C.M. Khoo, Metformin and colorectal cancer: a systematic review, meta-analysis and meta-regression, Int. J. Colorectal Dis. 35 (2020) 1501–1512. https://doi.org/10.1007/s00384-020-03676-x.

[17] L.P. Bharath, B.S. Nikolajczyk, The intersection of metformin and inflammation, Am. J. Physiol. Cell Physiol. 320 (2021) C873–C879. https://doi.org/10.1152/ajpcell.00604.2020.

[18] T.T. Nguyen, T.T. Ung, S. Li, S. Lian, Y. Xia, S.Y. Park, Y. Do Jung, Metformin inhibits lithocholic acid-induced interleukin 8 upregulation in colorectal cancer cells by suppressing ROS production and NF-kB activity, Sci. Rep. 9 (2019) 2003. https://doi.org/10.1038/s41598-019-38778-2.

[19] M. Moossavi, N. Parsamanesh, A. Bahrami, S.L. Atkin, A. Sahebkar, Role of the NLRP3 inflammasome in cancer, Mol. Cancer 17 (2018) 158. https://doi.org/10.1186/s12943-018-0900-3.

[20] T. Zhang, C. Ma, Z. Zhang, H. Zhang, H. Hu, NF-κB signaling in inflammation and cancer, MedComm 2 (2021) 618–653. https://doi.org/10.1002/mco2.104.

[21] A. Abderrazak, T. Syrovets, D. Couchie, K. El Hadri, B. Friguet, T. Simmet, M. Rouis, NLRP3 inflammasome: From a danger signal sensor to a regulatory node of oxidative stress and inflammatory diseases, Redox Biol. 4 (2015) 296–307. https://doi.org/10.1016/j.redox.2015.01.008.

[22] C.A. Dinarello, Interleukin-1 in the pathogenesis and treatment of inflammatory diseases, Blood 117 (2011) 3720–3732. https://doi.org/10.1182/blood-2010-07-273417.

[23] V. Singh, S. Ubaid, M. Kashif, T. Singh, G. Singh, R. Pahwa, A. Singh, Role of inflammasomes in cancer immunity: mechanisms and therapeutic potential, J. Exp. Clin. Cancer Res. 44 (2025) 109. https://doi.org/10.1186/s13046-025-03366-y.

[24] R. Ma, B. Yi, A.I. Riker, Y. Xi, Metformin and cancer immunity, Acta Pharmacol. Sin. 41 (2020) 1403–1409. https://doi.org/10.1038/s41401-020-00508-0.

[25] T. Higurashi, A. Nakajima, Metformin and Colorectal Cancer, Front. Endocrinol. 9 (2018) 622. https://doi.org/10.3389/fendo.2018.00622.

[26] I.I. Herbey, N.V. Ivankova, V.R. Katkoori, O.A. Mamaeva, Colorectal cancer and hypercholesterolemia: review of current research, Exp. Oncol. 27 (2005) 166–178.

[27] J.D. Potter, M.L. Slattery, R.M. Bostick, S.M. Gapstur, Colon Cancer: A Review of the Epidemiology, Epidemiol. Rev. 15 (1993) 499–545. https://doi.org/10.1093/oxfordjournals.epirev.a036132.

[28] L.A. Aaltonen, S.R. Hamilton, World Health Organization, International Agency for Research on Cancer, eds., Pathology and genetics of tumours of the digestive system, IARC Press ; Oxford University Press (distributor,), Lyon : Oxford, 2000.

[29] K. Friedman, A.S. Brodsky, S. Lu, S. Wood, A.J. Gill, K. Lombardo, D. Yang, M.B. Resnick, Medullary carcinoma of the colon: a distinct morphology reveals a distinctive immunoregulatory

microenvironment, Mod. Pathol. 29 (2016) 528–541. https://doi.org/10.1038/modpathol.2016.54.

[30] T. Nozoe, H. Anai, S. Nasu, K. Sugimachi, Clinicopathological characteristics of mucinous

carcinoma of the colon and rectum, J. Surg. Oncol. 75 (2000) 103–107. https://doi.org/10.1002/1096-9098(200010)75:2<103::AID-JSO6>3.0.CO;2-C.

[31] S. Kakar, T.C. Smyrk, Signet ring cell carcinoma of the colorectum: correlations between microsatellite instability, clinicopathologic features and survival, Mod. Pathol. 18 (2005) 244–249. https://doi.org/10.1038/modpathol.3800298.

[32] O. Marzouk, J. Schofield, Review of Histopathological and Molecular Prognostic Features in Colorectal Cancer, Cancers 3 (2011) 2767–2810. https://doi.org/10.3390/cancers3022767.

[33] A. Remo, M. Fassan, A. Vanoli, L.R. Bonetti, V. Barresi, F. Tatangelo, R. Gafà, G. Giordano, M. Pancione, F. Grillo, L. Mastracci, Morphology and Molecular Features of Rare Colorectal Carcinoma Histotypes, Cancers 11 (2019) 1036. https://doi.org/10.3390/cancers11071036.

[34] S. Zhao, C. Jang, J. Liu, K. Uehara, M. Gilbert, L. Izzo, X. Zeng, S. Trefely, S. Fernandez, A. Carrer, K.D. Miller, Z.T. Schug, N.W. Snyder, T.P. Gade, P.M. Titchenell, J.D. Rabinowitz, K.E. Wellen, Dietary fructose feeds hepatic lipogenesis via microbiota-derived acetate, Nature 579 (2020) 586–591. https://doi.org/10.1038/s41586-020-2101-7.

[35] M. Kyrgiou, I. Kalliala, G. Markozannes, M.J. Gunter, E. Paraskevaidis, H. Gabra, P. Martin-Hirsch, K.K. Tsilidis, Adiposity and cancer at major anatomical sites: umbrella review of the literature, BMJ (2017) j477. https://doi.org/10.1136/bmj.j477.

[36] Y. Ma, Y. Yang, F. Wang, P. Zhang, C. Shi, Y. Zou, H. Qin, Obesity and Risk of Colorectal Cancer: A Systematic Review of Prospective Studies, PLoS ONE 8 (2013) e53916. https://doi.org/10.1371/journal.pone.0053916.

[37] M. Daca Alvarez, I. Quintana, M. Terradas, P. Mur, F. Balaguer, L. Valle, The Inherited and Familial Component of Early-Onset Colorectal Cancer, Cells 10 (2021) 710. https://doi.org/10.3390/cells10030710.

[38] G. Perrod, G. Rahmi, C. Cellier, Colorectal cancer screening in Lynch syndrome: Indication, techniques and future perspectives, Dig. Endosc. 33 (2021) 520–528. https://doi.org/10.1111/den.13702.

[39] E.R. Fearon, B. Vogelstein, A genetic model for colorectal tumorigenesis, Cell 61 (1990) 759–767. https://doi.org/10.1016/0092-8674(90)90186-I.

[40] K. Tariq, K. Ghias, Colorectal cancer carcinogenesis: a review of mechanisms, Cancer Biol. Med. 13 (2016) 120–135. https://doi.org/10.28092/j.issn.2095-3941.2015.0103.

[41] M. Wright, J.S. Beaty, C.A. Ternent, Molecular Markers for Colorectal Cancer, Surg. Clin.

North Am. 97 (2017) 683–701. https://doi.org/10.1016/j.suc.2017.01.014.

[42] G. Peluso, P. Incollingo, A. Calogero, V. Tammaro, N. Rupealta, G. Chiacchio, M.L. Sandoval Sotelo, G. Minieri, A. Pisani, E. Riccio, M. Sabbatini, U.M. Bracale, C.A. Dodaro, N. Carlomagno, Current Tissue Molecular Markers in Colorectal Cancer: A Literature Review, BioMed Res. Int. 2017 (2017) 1–8. https://doi.org/10.1155/2017/2605628.

[43] E. Jang, D.C. Chung, Hereditary Colon Cancer: Lynch Syndrome, Gut Liver 4 (2010) 151–160. https://doi.org/10.5009/gnl.2010.4.2.151.

[44] S. Richman, Deficient mismatch repair: Read all about it (Review), Int. J. Oncol. 47 (2015) 1189–1202. https://doi.org/10.3892/ijo.2015.3119.

[45] M. Toyota, N. Ahuja, M. Ohe-Toyota, J.G. Herman, S.B. Baylin, J.-P.J. Issa, CpG island methylator phenotype in colorectal cancer, Proc. Natl. Acad. Sci. 96 (1999) 8681–8686. https://doi.org/10.1073/pnas.96.15.8681.

[46] D. Plundrich, S. Chikhladze, S. Fichtner-Feigl, R. Feuerstein, P.S. Briquez, Molecular Mechanisms of Tumor Immunomodulation in the Microenvironment of Colorectal Cancer, Int. J. Mol. Sci. 23 (2022) 2782. https://doi.org/10.3390/ijms23052782.

[47] B. Lakshmi Narendra, K. Eshvendar Reddy, S. Shantikumar, S. Ramakrishna, Immune system: a double-edged sword in cancer, Inflamm. Res. 62 (2013) 823–834. https://doi.org/10.1007/s00011-013-0645-9.

[48] D. Mittal, M.M. Gubin, R.D. Schreiber, M.J. Smyth, New insights into cancer immunoediting and its three component phases—elimination, equilibrium and escape, Curr. Opin. Immunol. 27 (2014) 16–25. https://doi.org/10.1016/j.coi.2014.01.004.

[49] G.P. Dunn, A.T. Bruce, H. Ikeda, L.J. Old, R.D. Schreiber, Cancer immunoediting: from immunosurveillance to tumor escape, Nat. Immunol. 3 (2002) 991–998. https://doi.org/10.1038/ni1102-991.

[50] G.P. Dunn, L.J. Old, R.D. Schreiber, The Three Es of Cancer Immunoediting, Annu. Rev. Immunol. 22 (2004) 329–360. https://doi.org/10.1146/annurev.immunol.22.012703.104803.

[51] J.B. Swann, M.J. Smyth, Immune surveillance of tumors, J. Clin. Invest. 117 (2007) 1137–1146. https://doi.org/10.1172/JCI31405.

[52] F. Cavallo, C. De Giovanni, P. Nanni, G. Forni, P.-L. Lollini, 2011: the immune hallmarks of cancer, Cancer Immunol. Immunother. 60 (2011) 319–326. https://doi.org/10.1007/s00262-010-0968-0.

[53] J.S. O'Donnell, M.W.L. Teng, M.J. Smyth, Cancer immunoediting and resistance to T cell-

based immunotherapy, Nat. Rev. Clin. Oncol. 16 (2019) 151–167. https://doi.org/10.1038/s41571-018-0142-8.

[54] W. Wiktor-Jedrzejczak, S. Gordon, Cytokine regulation of the macrophage (M phi) system studied using the colony stimulating factor-1-deficient op/op mouse, Physiol. Rev. 76 (1996)

927–947. https://doi.org/10.1152/physrev.1996.76.4.927.

[55] S. Gordon, P.R. Taylor, Monocyte and macrophage heterogeneity, Nat. Rev. Immunol. 5 (2005) 953–964. https://doi.org/10.1038/nri1733.

[56] C. Shi, E.G. Pamer, Monocyte recruitment during infection and inflammation, Nat. Rev. Immunol. 11 (2011) 762–774. https://doi.org/10.1038/nri3070.

[57] A. Parihar, T.D. Eubank, A.I. Doseff, Monocytes and Macrophages Regulate Immunity through Dynamic Networks of Survival and Cell Death, J. Innate Immun. 2 (2010) 204–215. https://doi.org/10.1159/000296507.

[58] K.J. Brempelis, I.N. Crispe, Infiltrating monocytes in liver injury and repair, Clin. Transl. Immunol. 5 (2016) e113. https://doi.org/10.1038/cti.2016.62.

[59] I. De Kleer, F. Willems, B. Lambrecht, S. Goriely, Ontogeny of Myeloid Cells, Front. Immunol. 5 (2014). https://doi.org/10.3389/fimmu.2014.00423.

[60] L. Cassetta, J.W. Pollard, Cancer immunosurveillance: role of patrolling monocytes, Cell Res. 26 (2016) 3–4. https://doi.org/10.1038/cr.2015.144.

[61] D.M. Whitelaw, M. Bell, The Intravascular Lifespan of Monocytes, Blood 28 (1966) 455–464. https://doi.org/10.1182/blood.V28.3.455.455.

[62] F. Geissmann, S. Jung, D.R. Littman, Blood Monocytes Consist of Two Principal Subsets with

Distinct Migratory Properties, Immunity 19 (2003) 71–82. https://doi.org/10.1016/S1074-7613(03)00174-2.

[63] V.G. Peddareddigari, D. Wang, R.N. DuBois, The Tumor Microenvironment in Colorectal Carcinogenesis, Cancer Microenviron. 3 (2010) 149–166. https://doi.org/10.1007/s12307-010-0038-3.

[64] K. Sawa-Wejksza, A. Dudek, M. Lemieszek, K. Kaławaj, M. Kandefer-Szerszeń, Colon cancer–derived conditioned medium induces differentiation of THP-1 monocytes into a mixed population of M1/M2 cells, Tumor Biol. 40 (2018) 101042831879788. https://doi.org/10.1177/1010428318797880.

[65] C. Li, X. Luo, Y. Lin, X. Tang, L. Ling, L. Wang, Y. Jiang, A Higher Frequency of

CD14+CD169+ Monocytes/Macrophages in Patients with Colorectal Cancer, PLOS ONE 10 (2015) e0141817. https://doi.org/10.1371/journal.pone.0141817.

[66] S. Missiroli, M. Perrone, C. Boncompagni, C. Borghi, A. Campagnaro, F. Marchetti, G. Anania, P. Greco, F. Fiorica, P. Pinton, C. Giorgi, Targeting the NLRP3 Inflammasome as a New Therapeutic Option for Overcoming Cancer, Cancers 13 (2021) 2297.

https://doi.org/10.3390/cancers13102297.

[67] Q. Deng, Y. Geng, L. Zhao, R. Li, Z. Zhang, K. Li, R. Liang, X. Shao, M. Huang, D. Zuo, Y. Wu, Q. Ma, NLRP3 inflammasomes in macrophages drive colorectal cancer metastasis to the liver, Cancer Lett. 442 (2019) 21–30. https://doi.org/10.1016/j.canlet.2018.10.030.

[68] X. Qiao, Z. Hu, F. Xiong, Y. Yang, C. Peng, D. Wang, X. Li, Lipid metabolism reprogramming in tumor-associated macrophages and implications for therapy, Lipids Health Dis. 22 (2023) 45. https://doi.org/10.1186/s12944-023-01807-1.

[69] N. Krahmer, R.V. Farese, T.C. Walther, Balancing the fat: lipid droplets and human disease, EMBO Mol. Med. 5 (2013) 973–983. https://doi.org/10.1002/emmm.201100671.

[70] Y. Wu, X. Pu, X. Wang, M. Xu, Reprogramming of lipid metabolism in the tumor microenvironment: a strategy for tumor immunotherapy, Lipids Health Dis. 23 (2024) 35. https://doi.org/10.1186/s12944-024-02024-0.

[71] Frizzled receptors and SFRP5 in lipid metabolism: Current findings and potential applications, in: Prog. Mol. Biol. Transl. Sci., Elsevier, 2023: pp. 377–393. https://doi.org/10.1016/bs.pmbts.2022.06.025.

[72] D. Wang, Q. Ye, H. Gu, Z. Chen, The role of lipid metabolism in tumor immune microenvironment and potential therapeutic strategies, Front. Oncol. 12 (2022) 984560. https://doi.org/10.3389/fonc.2022.984560.

[73] T.C. Walther, R.V. Farese, Lipid droplets and cellular lipid metabolism, Annu. Rev. Biochem. 81 (2012) 687–714. https://doi.org/10.1146/annurev-biochem-061009-102430.

[74] S. Vasseur, F. Guillaumond, Lipids in cancer: a global view of the contribution of lipid pathways to metastatic formation and treatment resistance, Oncogenesis 11 (2022) 46. https://doi.org/10.1038/s41389-022-00420-8.

[75] J. Luo, H. Yang, B.-L. Song, Mechanisms and regulation of cholesterol homeostasis, Nat. Rev. Mol. Cell Biol. 21 (2020) 225–245. https://doi.org/10.1038/s41580-019-0190-7.

[76] M.-S. Lee, S.J. Bensinger, Reprogramming cholesterol metabolism in macrophages and its role in host defense against cholesterol-dependent cytolysins, Cell. Mol. Immunol. 19 (2022) 327–336. https://doi.org/10.1038/s41423-021-00827-0.

[77] W.-Y. Hsieh, Q.D. Zhou, A.G. York, K.J. Williams, P.O. Scumpia, E.B. Kronenberger, X.P. Hoi, B. Su, X. Chi, V.L. Bui, E. Khialeeva, A. Kaplan, Y.M. Son, A.S. Divakaruni, J. Sun, S.T. Smale,

R.A. Flavell, S.J. Bensinger, Toll-Like Receptors Induce Signal-Specific Reprogramming of the Macrophage Lipidome, Cell Metab. 32 (2020) 128-143.e5.

https://doi.org/10.1016/j.cmet.2020.05.003.

[78] X.A.M.H. van Dierendonck, F. Vrieling, L. Smeehuijzen, L. Deng, J.P. Boogaard, C.-A. Croes, L. Temmerman, S. Wetzels, E. Biessen, S. Kersten, R. Stienstra, Triglyceride breakdown from lipid droplets regulates the inflammatory response in macrophages, Proc. Natl. Acad. Sci. U. S. A. 119 (2022) e2114739119. https://doi.org/10.1073/pnas.2114739119.

[79] J.J. Liang, I.D.C. Fraser, C.E. Bryant, Lipid regulation of NLRP3 inflammasome activity through organelle stress, Trends Immunol. 42 (2021) 807–823. https://doi.org/10.1016/j.it.2021.07.005.

[80] T. Liu, L. Zhang, D. Joo, S.-C. Sun, NF-κB signaling in inflammation, Signal Transduct. Target. Ther. 2 (2017) 17023. https://doi.org/10.1038/sigtrans.2017.23.

[81] R.A. Nolan, K.L. Reeb, Y. Rong, S.M. Matt, H.S. Johnson, K. Runner, P.J. Gaskill, Dopamine activates NF-κB and primes the NLRP3 inflammasome in primary human macrophages, Brain Behav. Immun. - Health 2 (2020) 100030. https://doi.org/10.1016/j.bbih.2019.100030.

[82] Y. He, H. Hara, G. Núñez, Mechanism and Regulation of NLRP3 Inflammasome Activation, Trends Biochem. Sci. 41 (2016) 1012–1021. https://doi.org/10.1016/j.tibs.2016.09.002.

[83] P. Duewell, H. Kono, K.J. Rayner, C.M. Sirois, G. Vladimer, F.G. Bauernfeind, G.S. Abela, L. Franchi, G. Nuñez, M. Schnurr, T. Espevik, E. Lien, K.A. Fitzgerald, K.L. Rock, K.J. Moore, S.D. Wright, V. Hornung, E. Latz, NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals, Nature 464 (2010) 1357–1361. https://doi.org/10.1038/nature08938.

[84] A.P. Perera, K. Sajnani, J. Dickinson, R. Eri, H. Körner, NLRP3 inflammasome in colitis and colitis-associated colorectal cancer, Mamm. Genome 29 (2018) 817–830. https://doi.org/10.1007/s00335-018-9783-2.

[85] I.C. Allen, E.M. TeKippe, R.-M.T. Woodford, J.M. Uronis, E.K. Holl, A.B. Rogers, H.H.

Herfarth, C. Jobin, J.P.-Y. Ting, The NLRP3 inflammasome functions as a negative regulator of tumorigenesis during colitis-associated cancer, J. Exp. Med. 207 (2010) 1045–1056. https://doi.org/10.1084/jem.20100050.

[86] J. Dupaul-Chicoine, A. Arabzadeh, M. Dagenais, T. Douglas, C. Champagne, A. Morizot, I.G. Rodrigue-Gervais, V. Breton, S.L. Colpitts, N. Beauchemin, M. Saleh, The Nlrp3 Inflammasome Suppresses Colorectal Cancer Metastatic Growth in the Liver by Promoting Natural Killer Cell Tumoricidal Activity, Immunity 43 (2015) 751–763.

https://doi.org/10.1016/j.immuni.2015.08.013.

[87] S. Del Barco, A. Vazquez-Martin, S. Cufí, C. Oliveras-Ferraros, J. Bosch-Barrera, J. Joven, B. Martin-Castillo, J.A. Menendez, Metformin: Multi-faceted protection against cancer, Oncotarget 2 (2011) 896–917. https://doi.org/10.18632/oncotarget.387.

[88] E.A.I.F. Queiroz, S. Puukila, R. Eichler, S.C. Sampaio, H.L. Forsyth, S.J. Lees, A.M. Barbosa, R.F.H. Dekker, Z.B. Fortes, N. Khaper, Metformin Induces Apoptosis and Cell Cycle Arrest Mediated by Oxidative Stress, AMPK and FOXO3a in MCF-7 Breast Cancer Cells, PLoS ONE 9 (2014) e98207. https://doi.org/10.1371/journal.pone.0098207.

[89] D. Ruggiero-Lopez, M. Lecomte, G. Moinet, G. Patereau, M. Lagarde, N. Wiernsperger, Reaction of metformin with dicarbonyl compounds. possible implication in the inhibition of advanced glycation end product formation, Biochem. Pharmacol. 58 (1999) 1765–1773. https://doi.org/10.1016/S0006-2952(99)00263-4.

[90] R. Giannarelli, M. Aragona, A. Coppelli, S. Del Prato, Reducing insulin resistance with metformin:

the evidence today, Diabetes Metab. 29 (2003) 6S28-6S35. https://doi.org/10.1016/S1262-3636(03)72785-2.

[91] G. Rena, E.R. Pearson, K. Sakamoto, Molecular mechanism of action of metformin: old or new insights?, Diabetologia 56 (2013) 1898–1906. https://doi.org/10.1007/s00125-013-2991-0.

[92] C. Mancusi, R. Izzo, G. Di Gioia, M.A. Losi, E. Barbato, C. Morisco, Insulin Resistance the Hinge Between Hypertension and Type 2 Diabetes, High Blood Press. Cardiovasc. Prev. 27 (2020) 515–526. https://doi.org/10.1007/s40292-020-00408-8.

[93] A. Kasprzak, Insulin-Like Growth Factor 1 (IGF-1) Signaling in Glucose Metabolism in Colorectal Cancer, Int. J. Mol. Sci. 22 (2021) 6434. https://doi.org/10.3390/ijms22126434.

[94] Y.-Y. Zhang, Y.-J. Li, C.-D. Xue, S. Li, Z.-N. Gao, K.-R. Qin, Effects of T2DM on cancer

progression: pivotal precipitating factors and underlying mechanisms, Front. Endocrinol. 15 (2024) 1396022. https://doi.org/10.3389/fendo.2024.1396022.

[95] A. Abdulla, H.Q. Sadida, J. Jerobin, I. Elfaki, R. Mir, S. Mirza, M. Singh, M.A. Macha, S. Uddin, K. Fakhro, A.A. Bhat, A.S.A.-S. Akil, Unraveling molecular interconnections and identifying potential therapeutic targets of significance in obesity-cancer link, J. Natl. Cancer Cent. 5 (2025) 8–27. https://doi.org/10.1016/j.jncc.2024.11.001.

[96] Z.-J. Zhang, Z.-J. Zheng, H. Kan, Y. Song, W. Cui, G. Zhao, K.E. Kip, Reduced Risk of Colorectal Cancer With Metformin Therapy in Patients With Type 2 Diabetes, Diabetes Care

34 (2011) 2323–2328. https://doi.org/10.2337/dc11-0512.

[97] M.N.A. Kamarudin, Md.M.R. Sarker, J.-R. Zhou, I. Parhar, Metformin in colorectal cancer: molecular mechanism, preclinical and clinical aspects, J. Exp. Clin. Cancer Res. 38 (2019) 491. https://doi.org/10.1186/s13046-019-1495-2.

[98] T.V. Kourelis, R.D. Siegel, Metformin and cancer: new applications for an old drug, Med. Oncol. 29 (2012) 1314–1327. https://doi.org/10.1007/s12032-011-9846-7.

[99] Y. Seo, J. Kim, S.J. Park, J.J. Park, J.H. Cheon, W.H. Kim, T.I. Kim, Metformin Suppresses Cancer Stem Cells through AMPK Activation and Inhibition of Protein Prenylation of the Mevalonate Pathway in Colorectal Cancer, Cancers 12 (2020) 2554. https://doi.org/10.3390/cancers12092554.

[100] X. Lai, B. Liu, Y. Wan, P. Zhou, W. Li, W. Hu, W. Gong, Metformin alleviates colitis-associated colorectal cancer via inhibition of the TLR4/MyD88/NFκB/MAPK pathway and macrophage M2 polarization, Int. Immunopharmacol. 144 (2025) 113683. https://doi.org/10.1016/j.intimp.2024.113683.