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THE INTERPLAY OF TLR9-MEDIATED AUTOPHAGY RESPONSE AND GROWTH FACTOR SIGNALING INHIBITION IN COLON ADENOCARCINOMA CELLS: CELL-FREE DNA EXPERIMENTS

PhD thesis

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LIST OF ABBREVIATIONS

Ago2: argonaute 2 protein
AIM2: absent in melanoma-2 receptor
AKBA: chemopreventive characteristics of 3-acetyl-11-keto-β-boswellic acid
AKT: Ak strain transforming
ALR: AIM2-like receptor
AMPK: AMP-activated protein kinase
ANOVA: analysis of variance
AP: activator protein
APC: antigen-presenting cell
ASC: apoptosis-associated speck-like protein including a C-terminal caspase recruitment domain
ATG: autophagy-related gene
ATG16L1: autophagy related 16 like 1
ATP: adenosine-triphosphate
Av: autophagic vacuole
BA145: powerful natural analog of AKBA
Bad: Bcl-2-associated agonist of cell death
BAFFR: B cell activating factor (BAFF) receptor
Bax: Bcl-2 associated X
Bcl-2: B-cell lymphoma 2
BECN1: Beclin-1
Caco-2: colon adenocarcinoma cell line
CASP1: procaspase 1 enzyme
CCDC25: coiled-coil domain containing protein-25
CD: cluster of differentiation
CD95L: CD95 (Fas) ligand
CD133: cluster of differentiation 133 /prominin 1, PROM1/
cfDNA: cell-free deoxynucleic acids
cGAS: cyclic guanosine monophosphate-adenosine monophosphate synthase
cGAS-STING: cyclic GMP-AMP synthase-signaling effector stimulator of interferon genes
c-Met: C mesenchymal epithelial transition factor /HGFR/
CpG: cytosolic cytosine-phosphate-guanine
CpG-ODN: CpG-oligodeoxynucleotide
CREB: cAMP-response element binding protein
CRC: colorectal cancer
DAMP: danger-associated molecular pattern
DC: dendritic cell
DISU: 4,4’-Diisothiocyanatostilbene-2,2’-disulfonic acid
DMSO: dimethyl sulfoxide
dsDNA: double-stranded DNA
EDTA: ethylenediaminetetraacetic acid
EGFR: epidermal growth factor receptor
EMT: epithelial–mesenchymal transition
ER: endoplasmic reticulum
ERK: extracellular signal-regulated kinase
ELK: erythroblast transformation specific (ETS) domain-containing protein
ET: extracellular trap
FAK: focal adhesion kinase
FBS: fetal bovine serum
Gab1: GRB2 associated binding protein 1
GAPDH: glyceraldehyde-3-phosphate dehydrogenase
g/f/mDNA: genomic/ fragmented/ hypermethylated self-DNA
GO: graphene oxide
Grb2: growth factor receptor-bound protein 2
GSDMD: linker region of gasdermin D
HBB: hemoglobin subunit beta
HCC: hepatic cell carcinoma
HDL: high-density lipoprotein

HER: human epidermal growth factor receptor
HGF: hepatocyte growth factor
HGFR: hepatocyte growth factor receptor /c-Met/
HIN: hematopoietic interferon-inducible nuclear
HMGB: DAMP protein high-mobility group B
HMGB1: high mobility group box 1
IGF1: insulin-like growth factor 1
IGF1R: insulin-like growth factor 1 receptor
IgM: immunoglobulin-M
IKBα/β: kinase complex (IKK)
IκB: kinase (IKK) complex that consists of two kinases (IKKα and IKKβ)
IKK: IκB kinase
IL: interleukin
IL-1r: interleukin-1 receptor
IFN: interferon
IRAK: interleukin receptor-associated kinase
IRF: interferon regulatory factor
IRS: insulin receptor substrates
JAK/STAT: Janus kinase/signal transducer and activator of transcription
JNK: C-jun N-terminal kinase
K^+: potassium ion
KRAS: Kirsten rat sarcoma virus
LAP: LC3-associated phagocytosis
LC3: microtubule-associated protein light chain 3
LRO: lysosome-related organelle
MAP1LC3: microtubule-associated protein 1 light chain 3
MALDI-TOF: matrix-assisted laser desorption-ionization time-of-flight mass spectrometry
MAPK: mitogen activated phosphokinase/protein kinase
MEK: mitogen-activated extracellular signal-regulated kinase
MHC I/II: major histocompatibility complex class I/II
mRNA: messenger RNA
mTOR: mammalian target of rapamycin
mTORC: mTOR complex
MUC-1: transmembrane glycoprotein mucin 1
MVB: multivesicular body
MyD88: myeloid differentiation primary response gene 88
NEAT1: nuclear paraspeckle assembly transcript 1
NEMO: nuclear factor-kappa B essential modulator
NET: neutrophil extracellular trap
NIK: nuclear factor-kappa B-inducing kinases
NIPT: non-invasive prenatal testing
NF-κB: nuclear factor kappa-B
NK: natural killer
NOD-SCID: nonobese diabetic-severe combined immunodeficiency
ODN: synthetic CpG-oligodeoxyribonucleotide
P: phosphor
PAMP: pathogen-associated molecular pattern
PBS: phosphate buffered saline
PCR: polymerase chain reaction
pDC: plasmacytoid dendritic cell
PD-L1: programmed death ligand 1
PI3K: phosphoinositide 3-kinase
PKC: protein kinase C
PM: plasma membrane
poly(I:C): polyinosinic:polycytidylic acid
PPP: picropodophyllin
PRR: pattern-recognition receptor
PYD: pyrin domain
RAC1: Ras-related C3 botulinum toxin substrate 1
RAF: rapidly accelerated fibrosarcoma
Ras: GTPase protein
RAS: rat sarcoma virus

RANK: receptor activator of nuclear factor-kappa B
RELB: RelB gene product transcription factor
RhD: rhesus D (blood type antigen/Rh+ or Rh-/)
RNA: ribonucleic acid
RTK: receptor tyrosine kinase
RT-PCR: reverse transcription-polymerase chain reaction
ROS: reactive oxygen species
S100: small acidic protein 100
SAA: serum amyloid A
Shc: adaptor protein
SIRT1: sirtuin 1
SOS: sinusoidal obstruction syndrome
SOX2: SRY-related HMG-box transcription factor 2
SphK1: sphingosine kinase 1
SQSTM1: sequestosome 1 /p62/
Src: SRC proto-oncogene, non-RTK
ssRNA: single stranded RNA
STAT: signal transducer and activator of transcription
STING: cGAS-stimulator of interferon genes
TAK1: transforming growth factor beta-activated kinase 1
TBK1: tank-binding kinase 1
TCR: T cell receptor
TEM: transmission electon microscope
Th1: type 1 T-helper
TIRAP: Toll-interleukin 1 receptor domain-containing adapter protein
TLR: Toll-like receptor
TNFα: tumor necrosis factor alpha
TP53: tumor protein p53
TRAF: tumor necrosis factor receptor-associated factor
TRAM: Toll-like receptor 4 adaptor protein
Treg: regulatory T cell
TRIF: Toll-interleukin-1 receptor-domain-containing adapter-inducing interferon
TRX: three prime repair exonuclease 1
Tukey HSD test: Tukey’s honestly significant difference test
ULK: Unc-51-like autophagy-activating kinases
UNC93B1: Unc-93 homolog B1 transmembrane protein
UVRAG: Beclin1-UV-irradiation resistance-associated gene
VEGFR: vascular endothelial growth factor receptor
VSV: vesicular stomatitis virus
WES: capillary Western blot

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1. INTRODUCTION

Mandel and Métais [1] are credited with the discovery of cell-free deoxynucleic acids (cfDNA) in the plasma of cancer patients in 1948. Subsequent research identified a correlation between the concentration of cfDNA and the development of systemic lupus erythematosus [2]. The use of cfDNA for tumor diagnostics began in 1977, but the limitations of the available technology determine its effectiveness [3]. In 1997, real-time polymerase chain reaction was introduced, which enabled the detection of RhD (blood type antigen) and fetal sex in maternal plasma [4]. In 2011, the introduction of massive parallel sequencing marked a significant advancement in non-invasive fetal genetic disease detection [5]. Today, approximately fifty percent of prenatal genetic examinations employ non-invasive prenatal testing (NIPT) [6]. Recent changes, like the rise in the number of liquid biopsies and the need for more screening, disease activity monitoring, and therapeutic response assessment, have made it easier for cfDNA research to get going again. Examination of the 5' end of the extracellular DNA revealed that it is not a junk molecule, which makes it unique [7]. However, research into the immunological properties of cfDNA, such as its possible immunomodulatory or therapeutic benefits, is still in its early stages. Nevertheless, studies are mainly focused on the function of cfDNA as a biomarker.

The objective of my PhD work was to provide an insight into the extremely complex immunobiological effects of cfDNA in colon adenocarcinoma cells.

1.1 The origin and characteristics of cfDNA

cfDNA is often present in different human body fluids, and although certain parts of its molecular source have been determined, there is an increasing corpus of research focused on investigating the unidentified factors that contribute to its development [8,9]. Various hypothetical internal sources and their corresponding processes have been proposed, excluding external sources of cfDNA. Regarding the source of cfDNA, it is feasible to distinguish between cancerous cells (such as tumor cells found locally and circulating, micrometastases, and cells within the tumor microenvironment) and non-cancerous cells (including muscle cells, epithelial cells, ovum cells, bone cells, myeloid cells, and lymphoid cells) [10].

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The mechanisms responsible for the release of cfDNA display significant variability. cfDNA is released by a number of processes, such as apoptosis, necrosis, pyroptosis, mitotic catastrophe, autophagy, phagocytosis, oncosis, NETosis, and DNA excision repair damage [11,12]. Alternatively, active release can also happen via macromolecular structures, including DNA-protein complexes, extracellular traps, micronucleation caused by genomic instability, extrachromosomal circular DNA, or microvesicles called exosomes [13-15]. These processes are illustrated in the **Figure 1**.

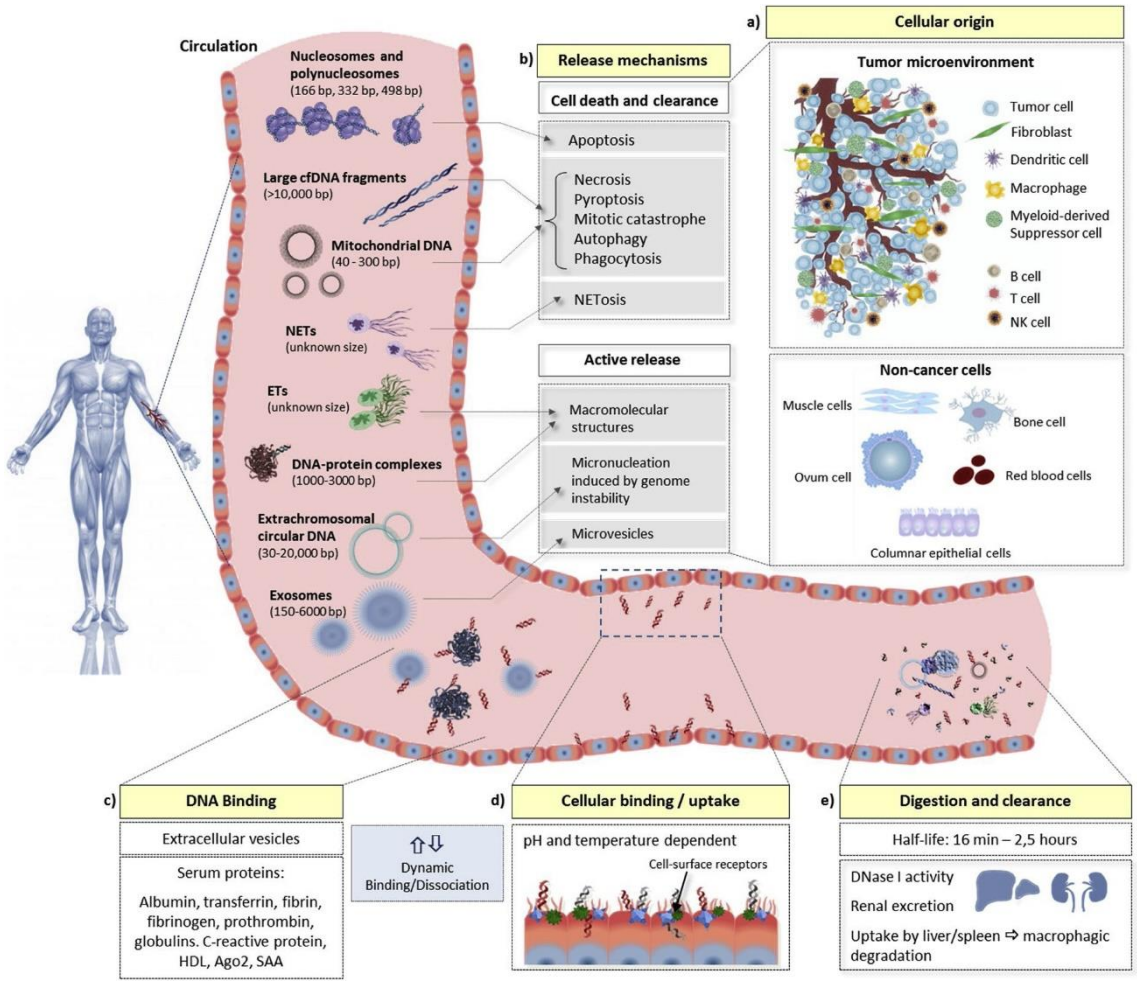

FIGURE 1 | The origin and characteristics of cfDNA [10].
a) Cell-free DNA (cfDNA) derives from various sources. DNA can be extracted from these cells by several mechanisms. b) The extracellular concentration of cfDNA is hence extremely contingent upon the velocity of its release from cells. Nevertheless, upon entering circulation, cfDNA levels are additionally affected by its. c) Dynamic interactions with extracellular vesicles and various serum proteins, d) Rates of binding, dissociation, and cellular internalization. e) Rates of degradation or clearance, encompassing the activity of DNAse I, renal excretion into urine and absorption by the liver and spleen.
Ago2: argonaute 2 protein; ETs: extracellular traps; HDL: high-density lipoprotein; NETs: neutrophil extracellular traps; NK cell: natural killer cell; SAA: serum amyloid A

Due to the absence of universally acknowledged methods, circulating human cfDNA quantification is still uncertain, and the data is inconsistent. Different factors, such as the matrix (plasma, serum, urine, cerebral fluid, etc.), the sample collection method (CellSave tubes or tubes with EDTA), the centrifugation variables (speed, temperature, duration), the isolation kits, and the storage conditions for cfDNA, can all change the results of a measurement [16]. Healthy people have lower cfDNA levels than diseased individuals. Recent research found that human plasma cfDNA content can exceed 500 ng/μL [17-20]. Advanced tumors [18-20], autoimmune [21-25], inflammatory [26], traumatic [27,28], post-transplantation [29], or viral diseases [30,31] exhibit higher levels. Additionally, significant physical exercise, such as half marathons, ultramarathons, and TRX workouts [32,33], and pregnancy [34], might raise cfDNA levels. As early as the first trimester, maternal blood contains 10–15% fetal cfDNA, mostly from placental trophoblast cells [35,36].

The concentration of cfDNA may rise not only due to the indicated circumstances but also as a result of an increased release. Inadequate clearance mechanisms may significantly contribute to the elevated levels of circulating cfDNA. Extracellular nuclease analogues, namely DNase I and DNase I-like III (DNase I L3), effectively perform the process of breaking down both unbound and protein-bound DNA [37]. Deviations in DNase I activity, such as decreased serum DNase I activity, elevated levels of DNase I inhibitors, and new mutations in the enzyme, could potentially impact the ability of the enzyme to identify and break down DNA [38-40]. Furthermore, several factors, such as DNA-interacting molecules [41], anti-DNase antibodies [42,43], and deficiencies in DNase I activating cofactors (such as complement component C1q [44], TREX1 Dnase [45], serum amyloid P component [46], IgM [47], C-reactive protein [48], and mannan-binding lectin), can also affect the function of the enzyme [49].

1.2 Recognition and immunomodulatory role of cfDNA

cfDNA has been shown in experiments to have immunomodulatory capabilities in addition to its role as a biomarker and diagnostic tool. It has the ability to influence the onset, progression, or reduction of inflammation. Maintaining self-tolerance necessitates the presence of self-DNA in both the nucleus and the mitochondria. However, under stressful circumstances, self-DNA can enter the cytosol due to nuclear or mitochondrial damage. The absence of infection appears to activate the inflammatory response, which is most likely started by the production of internal warning signals known as danger-associated molecular patterns (DAMPs). These DAMPs later trigger immune responses through pattern-recognition receptors (PRRs). cfDNA has the capacity to serve as a DAMP [50,51].

Several DNA-sensing receptors can detect cfDNA. These include cGAS, TLR9, and AIM2 receptors [52].

The cGAS recognizes cytosolic DNA molecules, activating IRF3 and synthesizing IFN-β or type 1 IFNs [53]. Due to their higher binding ability, cGAS detects extracellular nucleosomes better than double-stranded DNA (dsDNA) [53]. When cytosolic DNA is identified, cGAS activates *STING* [54,55]. The recognition length of dsDNA is 36 base pairs or more by the cGAS enzyme. Detecting this event activates cGAS-*STING*-mediated effectors, leading to the production of type 1 IFNs and other cytokines that rely on NF-κB, regardless of DNA sequence [50,56,57]. *STING* activates NF-κB, MAPK, and STAT6, resulting in autophagosome formation. To accomplish this, *STING* forms *LC3* and *Atg9a* punctas. The process starts when *STING* finds cytosolic DNA [58-61]. In response to cytosolic dsDNA, *BECN1* interacts with cGAS to limit cyclic GMP-AMP production. Disrupting cGAS-dsDNA interactions accomplishes this inhibition. *BECN1* releases Rubicon, an autophagy suppressor, when cGAS interacts with it. Class III *PI3K* release induces autophagy. Thus, autophagy eliminates cytosolic dsDNA [62]. Autophagy eliminates cfDNA effectively and reduces inflammation. When autophagy is impaired, several cytosolic PRRs identify cfDNA and induce inflammation [62], as shown in **Figure 2**.

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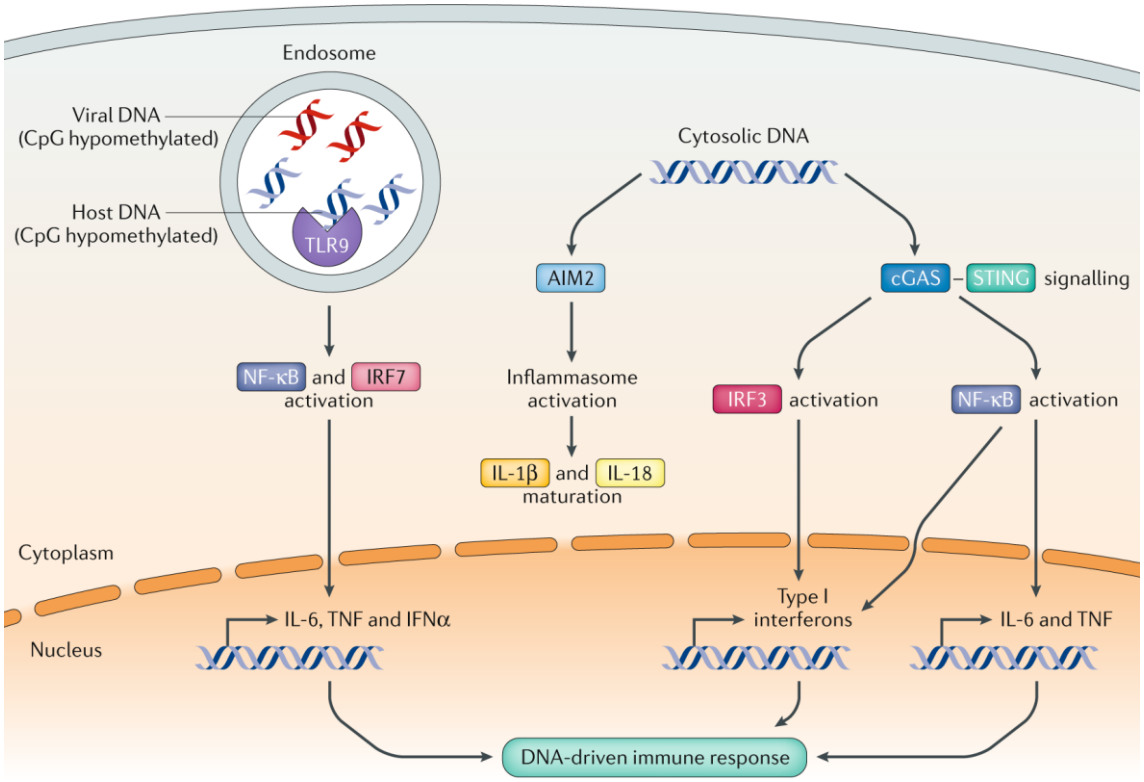

FIGURE 2 | DNA-sensing receptors [63].
The identification of pathogens by nucleic acid sensors is a fundamental aspect of innate immunity. RNA-sensing and DNA-sensing receptors detect foreign nucleic acids in subcellular compartments and, once identified, activate immunological signaling pathways to protect the host. The ability of native DNA to activate cGAS has been implicated as a crucial mechanism in triggering inflammation, and the cGAS-STING pathway has been implicated in the development of human inflammatory disorders and cancer.
AIM2:absent in melanoma-2 receptors; cGAS-STING: cyclic GMP-AMP synthase-signaling effector stimulator of interferon genes; CpG: cytosolic cytosine-phosphate-guanine; IFNα: interferon alpha; IL-1β/6/18: interleukin 1β/6/18; IRF3/7: interferon regulatory factor 3/7; NF-κB: nuclear factor kappa-B
TLR9: Toll-like receptor 9; TNF: tumor necrosis factor

TLR9 is detectable in the endoplasmic reticulum (ER) under normal physiological circumstances. However, when cytosolic CpG-DNAs or self-DNAs enter the endosome or endolysosome, TLR9 migrates to these cellular compartments and identifies them as significant DAMPs [64,65]. TLR9 activation initiates a signaling cascade that relies on *MyD88*. This pathway triggers the activation of IRF3, leading to the production of type 1 IFNs. Furthermore, it triggers the activation of *NF-κB*, which subsequently stimulates the synthesis of pro-inflammatory cytokines. These pathways contribute to the development of inflammation and inflammatory diseases [66]. The TIR domain of *MyD88* triggers the activation of IRAK-4 and IRAK-1, which are interleukin-1 receptor-associated kinases [67,68]. The protein IRAK-4 aids in *TRAF6* recruitment to induce TAK1 activation [69]. The TAK1 enzyme promotes the addition of K63-linked ubiquitin molecules to NEMO,

which in turn enables the phosphorylation of the IKK complex. This mechanism is essential in the signaling pathways of NF-κB, IRF3, and MAPK [70]. TLR9 possesses the capacity to differentiate between two unique classifications of DNA, specifically DNA derived from pathogens and DNA produced from oneself. The study revealed that the interaction and stimulation of TLR9 by synthetic CpG-ODNs and cfDNAs are affected by parameters such as their nucleotide sequence, length, and ability to form dimers [71-73]. Intracellular compartmentalization of TLR9 is a process that helps distinguish between self-DNA and non-self-DNA, which comes from external sources. Binding leads to an increase in dimerization and subsequent activation [74].

Platelets have been shown to feature PRRs that can be triggered when they interact with DAMPs [75]. Both murine and human platelets express TLR9 [76,77], which is important since platelets play a crucial role in integrating innate and adaptive immune responses as well as their fundamental function in hemostasis [75]. Platelet activation triggers the production of P-selectin by platelets, which enables them to stick to many cell types, including granulocytes. As a result, this contact stimulates granulocyte activation, which then migrates to tissue damage sites. cfDNA activates platelets, which results in the formation of neutrophil extracellular traps (NETs) [75].

ALR protein becomes activated when it identifies and attaches to self-DNA molecules that enter the cytosol due to cellular injury or exosomes [78]. AIM2 is efficiently activated when it detects self-DNA, which is 80-300 base pairs long [79,80]. AIM2's HIN domain can find cytosolic DNA because it is expressed in hematopoietic cells, can be activated by interferon, and is located in the nucleus. To enhance inflammasome complex formation, AIM2's PYD interacts with ASC's PYD. This complex converts pro-CASP1 into CASP1 [79-81]. CASP1 fragments and releases pro-inflammatory cytokines IL-1 and IL-18 from their precursors [81]. CASP1 breaks GSDMD's linker region, releasing IL-1 and IL-18 from cells. The flow of K^+ out of the GSDMD pore stops cGAS activity and the production of type 1 IFN through the *STING* pathway. The outflow of K^+ also initiates pyroptosis [82-84]. cGAS-*STING* promotes type 1 IFN production, which AIM2-generated GSDMD inhibits [78]. Another finding is that the AIM2-ASC inflammasome inhibits *STING-TBK1*, which activates IRF3 and releases type 1 IFNs. AIM2 is quiescent without particular cytosolic DNA [85]. **Figure 3** illustrates these mechanisms.

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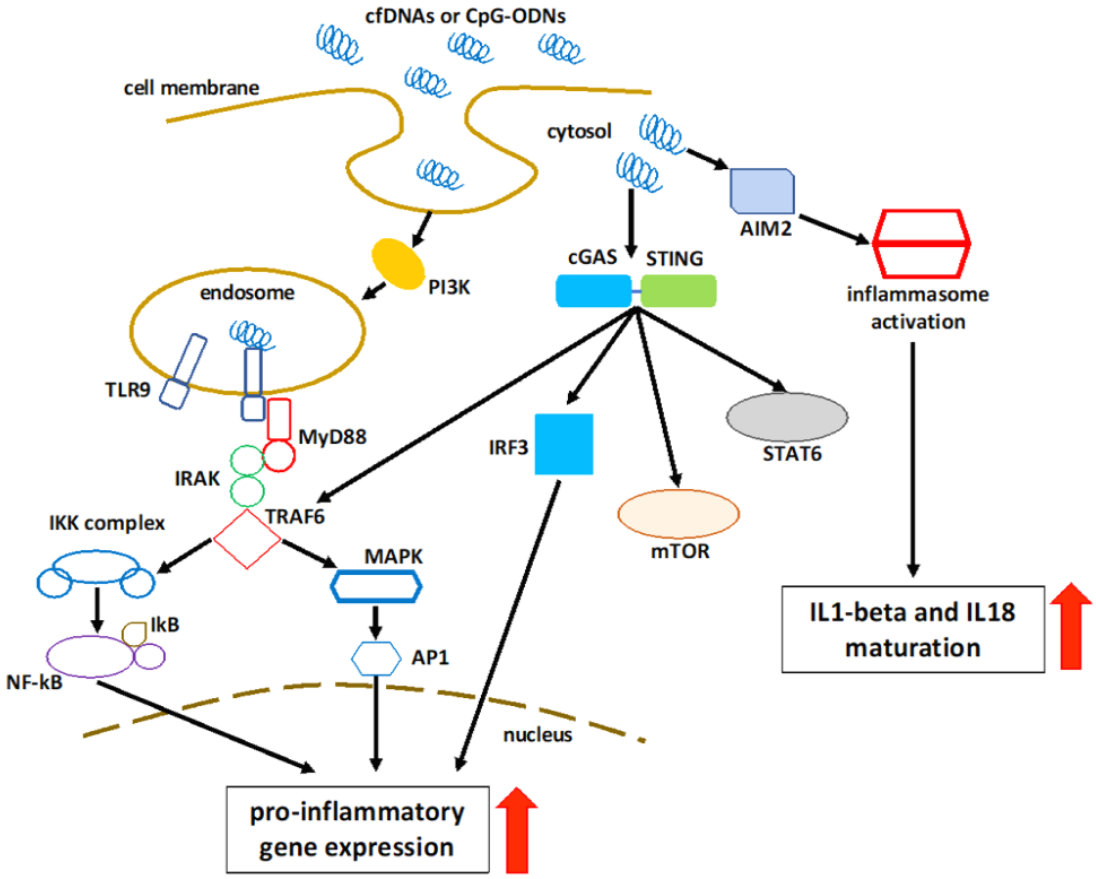

FIGURE 3 | Schematic representation of the cfDNA detection process and the subsequent activation of pathways [86].
The role of Class III PI3K in the internalization of cfDNA and CpG-ODNs into endosomal vesicles that contain TLR9 is apparent. The intracellular activation signal is sent by the interaction between cfDNA and TLR9. The MyD88 protein is specifically attracted to the Toll-interleukin-1 receptor domain of TLR9, resulting in the subsequent activation of the IRAK-TRAF6 complex. This mechanism triggers the activation of both the mitogen-activated protein kinase (MAPK) and inhibitor of IKK (IκB kinase) complexes, leading to an increase in transcription factors such as NF-κB and activator protein 1 (AP1). The activation of a *STING*-dependent immune response is initiated by the detection of DNA in the cytoplasm, which occurs through the cGAS-mediated pathway. The *cGAS-STING* pathway may activate several signaling molecules, such as IRFs, mTOR, STAT6, and MAPK, through both direct and indirect pathways. The AIM2 protein in the cytosol has a strong attraction to double-stranded DNA, resulting in the creation of a molecular complex called the AIM2 inflammasome. Consequently, the activation of caspase 1 occurs, which leads to the production of pro-inflammatory cytokines IL1-beta and IL18, finally causing pyroptosis.
AIM2: absent in melanoma-2 receptor; AP1: activator protein 1; cfDNA: cell-free deoxynucleic acids; cGAS-STING: cyclic GMP-AMP synthase-signaling effector stimulator of interferon genes; CpG-ODN: CpG-oligodeoxynucleotide; IL1-beta/18: interleukin 1-beta/18; IκB: kinase (IKK) complex that consists of two kinases (IKKα and IKKβ); IRAK: interleukin receptor-associated kinase; IRF3: interferon regulatory factor 3; MAPK: mitogen activated phosphokinase/protein kinase; mTOR: mammalian target of rapamycin; MyD88: myeloid differentiation primary response gene 88; NF-κB: nuclear factor kappa-B; PI3K: phosphoinositide 3-kinase; STAT6: signal transducer and activator of transcription 6; TLR9: Toll-like receptor 9; TRAF6:tumor necrosis factor receptor-associated factor 6

1.2.1 Description and features of TLRs and TLR9 signaling

TLRs are type 1 transmembrane glycoproteins that have Toll/interleukin-1 receptor signaling domains and extracellular leucine repeats. The first receptor discovered was TLR4, and a total of 10 human TLRs and 13 mouse TLRs have been reported [87]. Innate and adaptive immune cells, including monocytes, macrophages, lymphocytes, mast cells, and dendritic cells, typically harbor TLRs. However, transformed epithelial cells can express TLR4, TLR5, and TLR9 [88]. Bacterial DNA fragments activate apical epithelial TLR9 to maintain colonic homeostasis [89].

TLRs recognize DNA, RNA, and microbial cell wall components. TLR1, TLR2, TLR4, TLR5, and TLR6 reside on the cell membrane, while TLR3, TLR7, TLR8, and TLR9 are primarily within [63,90-92]. TLR receptors are drawn to patterns seen in bacteria, fungi, protozoa, and viruses [93,94]. Lipids and lipopeptides (TLR1, -2, -4, -6), bacterial flagellin (TLR5), and nucleic acid fragments activate TLRs. TLR3 attracts viral dsRNA, while TLR7 and TLR8 can detect ssRNA. TLR7 can also identify immunoglobulin-self-RNA complexes in autoimmune diseases. Imiquimod binds to TLR7. Bacterial, viral, immunoglobulin-DNA complexes, and synthetic ODNs with unmethylated CpG sequences activate TLR9 [93,94].

TLRs signal innate and adaptive immune responses. Dysregulated adaptive and innate immune activation, amplified by immune evasion of tumor cells, leads to cytotoxic consequences. This can eliminate unhealthy cells or slow cancer growth. TLRs detect microbial PAMPs. They can engage endogenous ligands, such as DAMPs [95]. By stimulating pDCs and macrophages, bacterial DNA and synthetic ODNs activate the innate and adaptive immune systems [96].

TLR9 activation causes pDCs to produce IFN-α. After IFN-α regulates their synthesis, B cells secrete pro-inflammatory (IL-6 and TNFα) and anti-inflammatory (IL-10) cytokines. MHC II surface antigens are also produced upon activation [96,97].

TLR9 activation is a complex and multi-step process. The uptake process is the least understood of the steps and varies depending on the fragment composition of the DNA. Many cell types can efficiently uptake single-stranded DNA. Since endosomes contain TLR9, cationic lipids can boost the uptake of double-stranded DNA [96,97]. Non-specific endocytosis helps transport fluorescein-isothiocyanate-labeled CpG DNA to the intracellular compartment, according to the study. Since DNA sequences without CpG

dinucleotides activate TLR9, the transport mechanism is unspecific. Non-CpG sequences can also limit immune activation in competition [97]. Entering the intracellular compartment causes endosomal acidic maturation. pH-raising drugs like chloroquine and bafilomycin A1 can slow this action. The production of pro- and anti-inflammatory cytokines increases B-cell proliferation [97].

The signal molecules, including MyD88, TRAF6, IRAK-1, and -4, as well as the p50/p65 heterodimer of NF-κB, lack specificity but are also involved in the signaling of other TLRs. The secretion of IFNs can also take place via a pathway associated with MAPK, which is now being thoroughly studied [98]. **Figure 4** summarises these processes.

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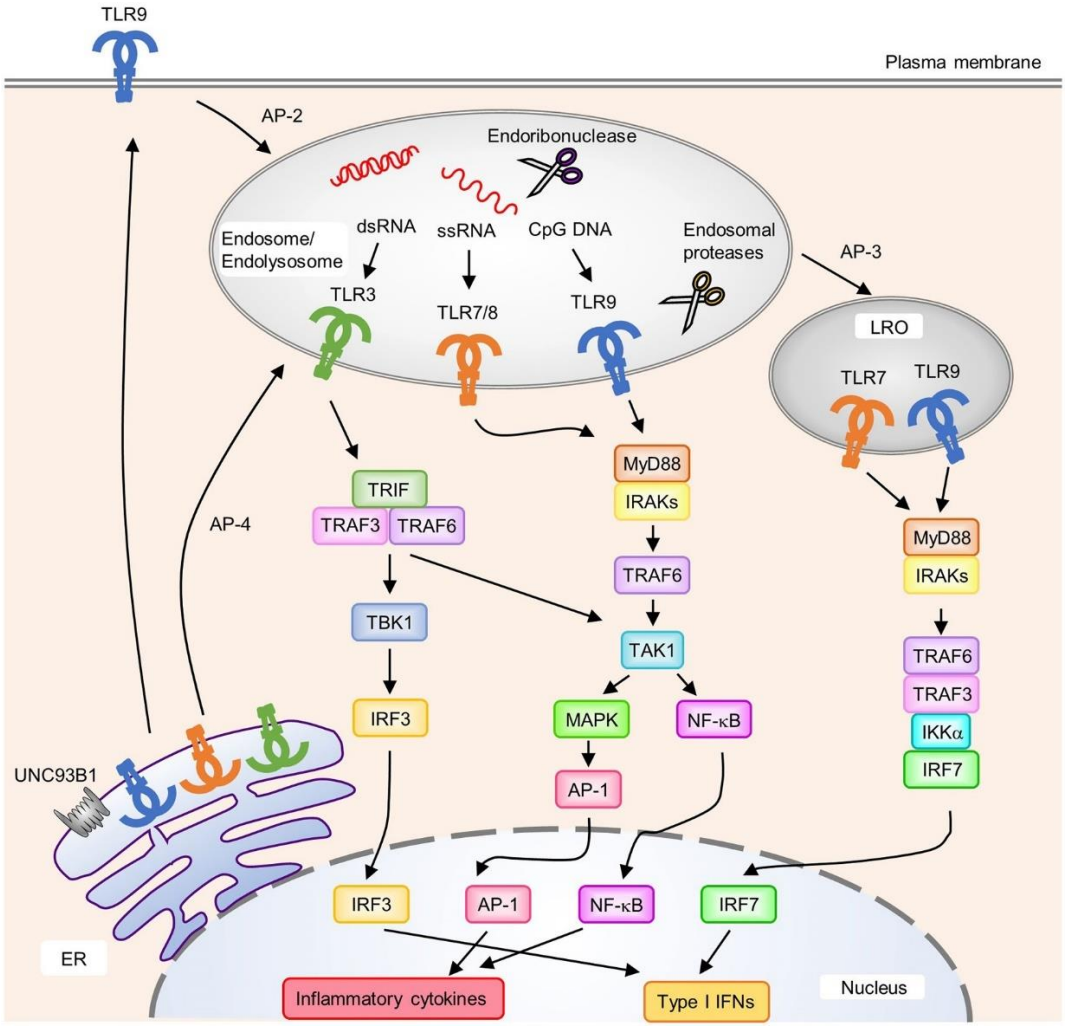

FIGURE 4 | Localization, intracellular transport, and signaling mechanisms of nucleic acid-sensing TLRs [99].

TLRs are produced in the ER and subsequently delivered to endosomes by UNC93B1. TLR9 requires the AP-2 complex to translocate from the cell surface to endosomes, whereas TLR7 interacts with the AP-4 complex to direct trafficking to endosomes. When cognate ligands are recognized, TLR7, TLR8, and TLR9 recruit MyD88 to activate downstream signaling pathways. MyD88 recruits IRAKs and TRAF6, which subsequently activate TAK1. Activated TAK1 activates AP-1 through MAPK, which initiates the transcription of pro-inflammatory cytokines. TAK1 also activates NF-κB, which in turn triggers the production of pro-inflammatory cytokines. It is thought that TLR7 and TLR9 in LRO make IRF7 active by joining with TRAF6, TRAF3, IKKα, and IRF7. This causes type I IFNs to be released. An AP-3 complex is required for the localization of TLR7 and TLR9 to LRO. TLR3 recruits TRIF to initiate downstream signaling pathways. To activate TBK1 and TAK1, TRIF recruits TRAF3 and TRAF6. TBK1 that is activated makes type I IFNs through IRF3, and TAK1 activated by NF-κB and AP-1 makes pro-inflammatory cytokines.

AP-1/2/3/4: Activator protein 1/2/3/4; CpG: cytosolic cytosine-phosphate-guanine; dsDNA: double stranded DNA; ER: endoplasmatic reticulum; IFNs: interferons; IKKα: IκB kinase alpha; IRAKs: interleukin receptor-associated kinases; IRF3/7: interferon regulatory factor 3/7; LRO: lysosome-related organelle; MAPK: mitogen activated phosphokinase/protein kinase; MyD88: myeloid differentiation primary response gene 88; NF-κB: nuclear factor kappa-B; ssRNA: single stranded RNA; TAK1: transforming growth factor beta-activated kinase 1; TBK1: tank-binding kinase 1; TLRs: Toll-like receptors; TRAF3/6: tumor necrosis factor receptor-associated factor 3/6; TRIF: Toll-interleukin-1 receptor-domain-containing adapter-inducing interferon; UNC93B1: Unc-93 homolog B1 transmembrane protein

1.2.1.1 Types of CpG-ODNs

Mouse and human cell line investigations have indicated that unmethylated CpG sequences stimulate the immune system [100,101]. Three types of CpG DNA sequences are based on their chemical composition and immune cell-mediated responses. The chemical makeup of these sequences is crucial to their immunostimulatory effect on immune cells [102]. Liu and colleagues [102] studied how the three types of CpG-ODN affect the immune system's responses to antigens in different ways in mouse models. It was found that both B- and C-class CpG-ODNs caused a strong Th1-mediated immune response, with similar antibody and CD4+/CD8+ T cell responses. The A-class CpG-ODNs raised the cytotoxicity and antibody levels of CD8+ T cells, but they did not change the IgG1/IgG2a ratio or increase the number of CD4+ and CD8+ T cells that produced IFN-γ. Based on this, three CpG-ODN groups showed various levels of targeted protection against *Listeria monocytogenes*, an intracellular bacterium. These three CpG-ODN groups had similar effects on IL-12 production. This study may help to understand the adjuvant properties of three CpG-OND groups. These findings may also aid the CpG-ODN adjuvant strategy [103,104].

Currently, clinical trials have examined the therapeutic use of TLR9 agonists in several forms of cancer, such as colon, pancreatic, and breast malignancies [105-108]. Furthermore, continuous research is underway to evaluate the effectiveness of TLR9 agonist therapy on esophageal squamous cell cancer [109], melanomas [110], lymphomas [111,112], non-small cell lung carcinomas [113], renal malignancies, and androgen-resistant prostate cancers [114]. Mechanism of action of CpG in cellular processes are irrustrated on **Figure 5**.

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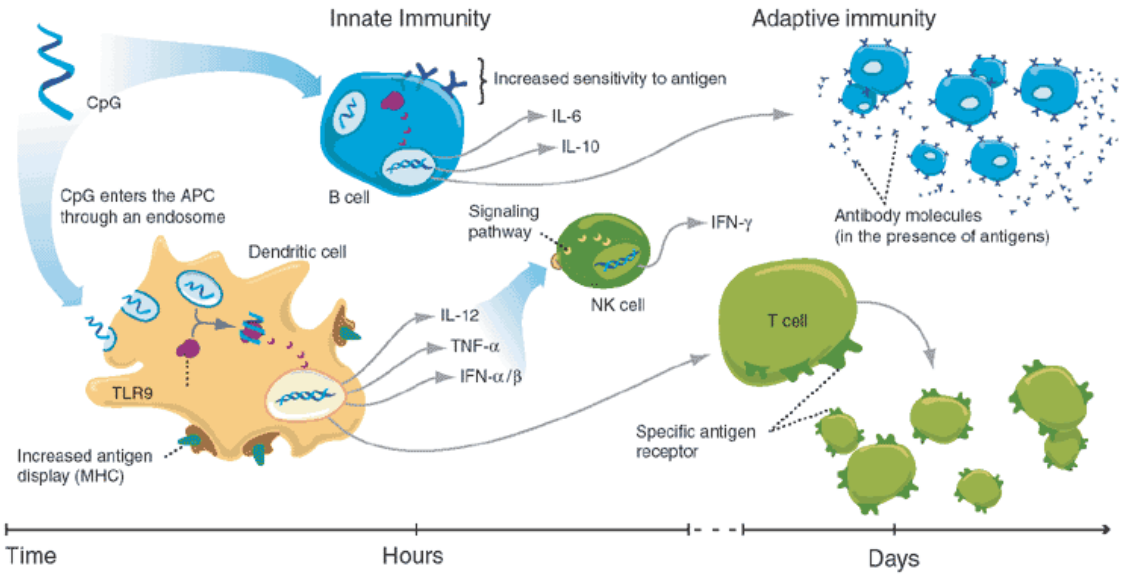

FIGURE 5 | Mechanism of action of CpG in cellular processes [115].
Most cell types take up DNA containing one or more CpG motifs through endocytosis, but only cells expressing the TLR9 receptor (B cells, plasmacytoid DCs (pDC), and several epithelial cells in humans) can activate it. TH1-like cytokine milieu is then made by these cells releasing IFN-α, IFN-β, IL-12, IP-10, and other cytokines and chemokines that support TH1. The secondarily activated NK cells secrete IFN; additionally, their antigen receptor increases the sensitivity of B cells to activation, and both B cells and plasmacytoid DCs express more costimulatory molecules, enhancing their capacity to trigger T-cell responses.
APC: antigen-presenting cell; CpG: cytosolic cytosine-phosphate-guanine; IFN-α/β/γ: interferon alpha/beta/gamma; IL-6/10/12: interleukin 6/10/12; MHC: major histocompatibility complex; NK: natural killer; TNFα: tumor necrosis factor alpha; TLR9: Toll-like receptor 9

1.2.2 TLR9 in inflammation and malignancy

TLRs 3, 4, 5, 7 and 8 have been detected in colorectal cancers [116]. Human colon cancer cells HCT15, SW620, and HT29 express of several TLRs, including TLR7-9 [117-119]. The increased expression of TLRs in tumor cells appears to play a role in tumor growth by improving their capacity to survive and move within the tumor microenvironment, which is characterized by ongoing inflammation and PAMPs [120]. However, prior studies have shown that boosting TLRs and their associated mediators, such as type I IFNs, may have the ability to modify the balance between immunological tolerance and anti-tumor responses. Therefore, researchers have hypothesized a controversial role for *TLR* signaling pathways in cancer cells[121].

TLRs may promote tumors by conveying pro-inflammatory, anti-apoptotic, proliferative, or pro-fibrogenic signals to tumor cells or the tumor environment. TLRs are critical for inflammatory signaling via *MyD88*-dependent and *MyD88*-independent

pathways. The *NF-κB* pathway is critical for TLRs' tumor-promoting effects. TLR activation increases the production of inflammatory cytokines, such as IL-1β, TNFα and IL-6, which contribute to tumor growth. The increase occurs through the *NF-κB* pathway [121-123]. Apoptosis suppression involves the *TLR* signaling pathway. The *NF-κB* pathway is a key regulator of apoptosis, controlling gene expression and restricting pathways that induce it [124,125].

Tumor cells cannot frequently deliver antigens; therefore, they rely on specialized APCs like DCs to create effective immune responses. Cancer scientists are interested in DCs because they can induce significant anti-tumor immune responses. Cancer cell inhibitory signals frequently cause a lack of DC activation, which can lead to immunological tolerance by eliminating T cells or promoting Tregs [126]. This, in turn, promotes tumor growth. DCs triggered by TLR signaling can deliver antigens, activate T lymphocytes and directly kill tumor cells [127,128]. TLR5 activation on DCs and TLR9 stimulation of pDCs boost immune responses to cancer [129,130]. Signal transduction based on DNA sequence and methylation pattern activates TLR9. Nucleic acid structure affects their immunomodulatory capabilities, including their ability to activate or repress immune responses and promote or inhibit tumor development [119,131]. Synthesized CpG-ODN agonists have been shown to activate TLR9 and fight colon cancer in mouse xenograft models. According to research, TLR9 agonists can increase type I IFN production in DCs, resulting in cytotoxic DCs. This activates NK and cytotoxic T cells, causing a strong immune response to cancer [132,133]. These processes are shown in **Figure 6**.

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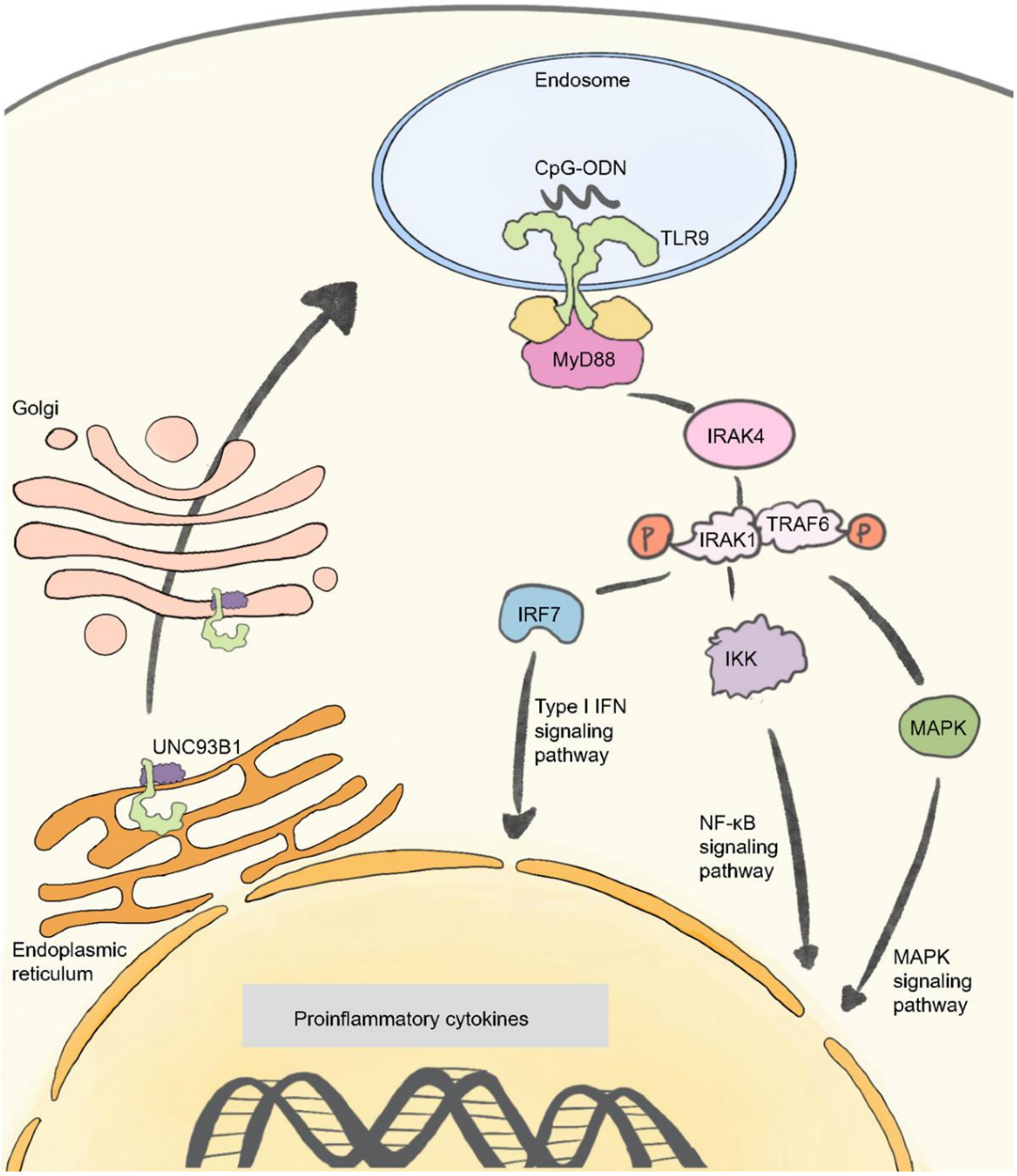

FIGURE 6 | The signaling pathways of TLR9 [134].
The binding of CpG-ODN to TLR9 activates the type I IFN signaling pathway, the NF-κB signaling pathway, and the MAPK signaling pathway, which promotes the expression and secretion of pro-inflammatory cytokines in target cells.
CpG-ODN: CpG-oligodeoxynucleotide; IFN: interferon; IKK: IκB kinase; IRAK1/4: interleukin receptor-associated kinase 1/4; IRF7: interferon regulatory factor 7; MAPK: mitogen activated phosphokinase/protein kinase; MyD88: myeloid differentiation primary response gene 88; NF-κB: nuclear factor kappa-B; TRAF6: tumor necrosis factor receptor-associated factor 6; TLR9: Toll-like receptor 9; UNC93B1: Unc-93 homolog B1 transmembrane protein

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1.3 Characteristics and process of autophagy

Autophagy, a well-conserved biological mechanism, encompasses numerous phases across the proteasomal breakdown route. Autophagy is a process that aids in the breakdown of excessive, damaged, or aged proteins and intracellular organelles. This is achieved by enclosing them within double-membraned vesicles called autophagosomes. Autophagosomes fuse with lysosomes/endosomes; their membranes break down and form autolysosomes [135].

Different types of autophagy have been distinguished according to the method by which cellular components are carried to lysosomes as well as their main physiological functions. The types of autophagy encompass macroautophagy, microautophagy, and chaperon-mediated autophagy. We can categorize autophagy into specific categories like lipophagy, ribophagy, nucleophagy, and mitophagy. These types entail the targeted breakdown of cytosolic proteins, lipids, and organelles such as ribosomes, nucleosomes, and mitochondria [135,136].

The term "macroautophagy," henceforth referred to as "autophagy," describes the non-discriminatory degradation of subcellular structures inside the cytoplasm [137]. These processes are illustrated in **Figure 7**.

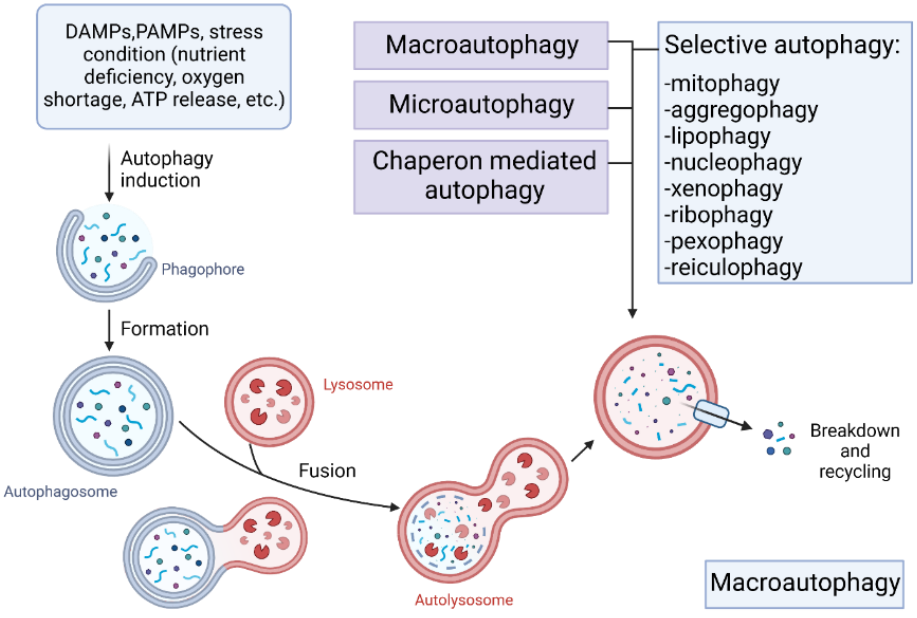

FIGURE 7 | The process of macroautophagy and the types of autophagy [138].
The image shows the formation of the autophagosome and the types of autophagy. (Created with BioRender.com)
ATP: adenosine-triphosphate; DAMP: danger-associated molecular pattern; PAMP: pathogen-associated molecular pattern

A few autophagy genes (*ATGs*) tightly regulate the complicated catabolic system, which involves multiple morphological steps. The encapsulation of molecules or particles for retention triggers the production of phagophores. Phagophores elongate and mature into autophagosomes. Finally, autophagosomes combine with lysosomes [135,137]. Multiple *ATG*-proteins, including the Ser/Thr kinases *ULK1/2 (ATG1*), a complex of lipid kinases, and two additional ubiquitin-like conjugation systems, enable the multiple dynamic functions listed above [139]. After that, *BECN1*, the mammalian equivalent of *Atg6* in yeast, and the *ATG14* genes control the phagophore. The inhibitory class 1 canonical *PI3K/AKT/mTOR* pathway and the promoting *JNK1* pathway are additional regulators. The ATG5-ATG12 complex, supported by *ATG16L1*, controls autophagosome formation. Additionally, *LC3/ATG8* is essential for autophagosome maturation. The ubiquitin-like systems *ATG10, ATG7*, and *ATG3* strictly regulate these processes. The LC3 protein and *UVRAG* gene regulate cargo engulfment, autophagosome closure and lysosomal fusion [140,141].

LC3, unlike other autophagy pathway components, may degrade particles without producing a double membrane. This accelerates phagosome formation. Alternative autophagy signaling (LAP) is called noncanonical autophagy [142]. Stress-related cell death processes, including intrinsic and extrinsic apoptosis and autophagy, can interact in a complex way. The fate of a cell depends on these pathways' interaction and function [143]. The *ATG6/Beclin-1* and *Bcl-2/Bcl-xL* interact to modulate their communication, with *Bcl-2* suppressing autophagy. TLR adaptors such as *MyD88* and *TRIF* can dissociate this complex. Activating the MAPK-JNK cascade or translocating HMGB-1 can also achieve this [140,143]. Autophagy and *NF-κB* signaling pathways interact in several ways, including positive and negative feedback loops, as **Figure 8** illustrates [141]. The tumor suppressor *p53* gene also controls autophagy. Depending on whether *p53* is in the nucleus or cytoplasm, it can activate or suppress autophagy [140,141].

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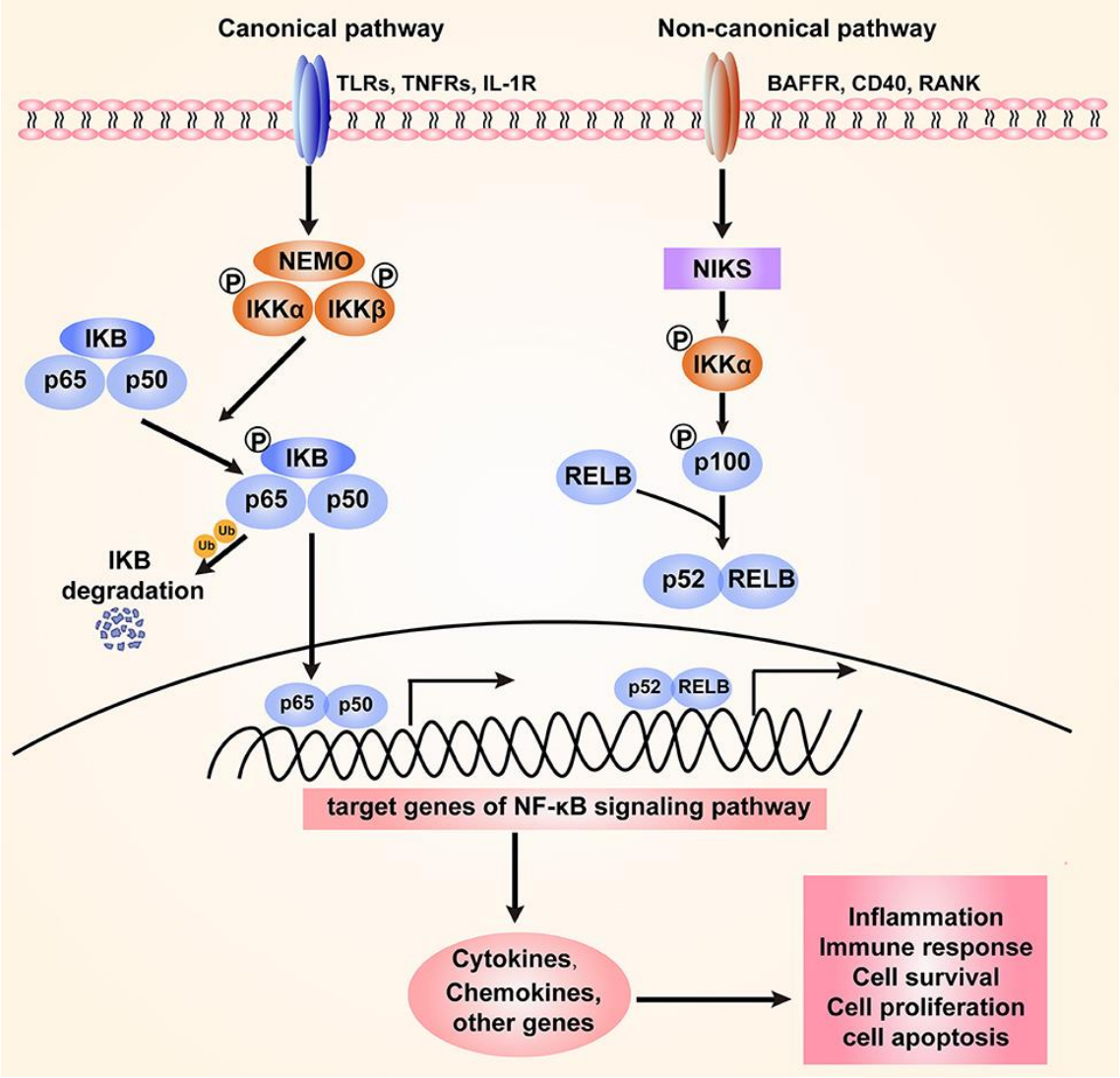

FIGURE 8 | The canonical and non-canonical NF-κB signaling pathways [144].
TLRs, TNFRs, and IL-1R activate the canonical pathway. This cascade activation results in the phosphorylation and destruction of the inhibitory protein IκB. Dissociation from the IB-containing complex activates NF-B, which then translocates into the nucleus. The non-canonical route relies on the activation of the NF-κB2 (p100)/RelB complex by BAFFR, CD40, and RANK. This cascade triggers the phosphorylation of NIK, which in turn phosphorylates IKKα. This activation leads to the translocation of the p52-RelB heterodimer to the nucleus. NF-κB signaling can change many cellular functions by affecting the expression of genes that code for cytokines, chemokines, and other things.
CD40: cluster of differentiation 40; BAFFR: B cell activating factor (BAFF) receptor; IL-1R: interleukin-1 receptor; IκB: kinase complex (IKK) that consists of two kinases (IKKα and IKKβ); IKKs: IκB kinases; NEMO: nuclear factor-kappa B essential modulator; NF-κB: nuclear factor kappa-B; NIK: NF-κB-inducing kinases; RANK: Receptor Activator of Nuclear Factor-kappa B; RELB: RelB gene product transcription factor; TLRs: Toll-like receptors; TNFRs: tumor necrosis factor receptor

Autophagy regulates cellular development, specialization, survival, and aging [145]. In addition, it affects inflammation and innate and adaptive immunological responses. Autophagy is essential and adaptable to cellular homeostasis. Several metabolic stress circumstances, such as lack of food and growth factor availability, can activate autophagy

to guarantee cell survival. Basal autophagy disruptions can lead to toxic chemical buildup and DNA damage, causing genomic instability. Most induced autophagy abnormalities impair cell survival [137,145].

Defective autophagy, which harms cells, has been related to cancer, neurological disorders, liver illnesses, viral diseases, aging and inflammatory conditions, including Crohn's disease [136,145-147].

Autophagy's dualistic "Janus" function is thought to have a role in carcinogenesis, as shown in **Figure 9**. This is because it can affect cancer cell survival and multiplication, especially in difficult conditions. Additionally, it can activate signaling pathways that kill cancer cells. Autophagy's effect on cellular defense or tumor cell growth depends on various internal and external factors. Specific tissue types, cellular environment, genetic makeup, and tumor advancement stage all have an impact on tumor growth. However, the relationship between autophagy and cancer networks is still unclear [145,146,148].

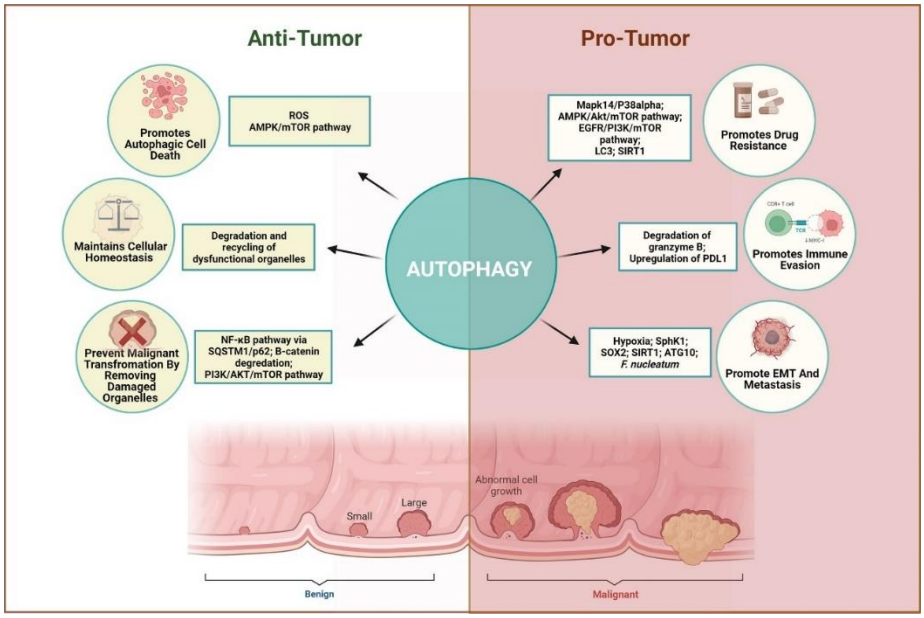

FIGURE 9 | The dual role of autophagy in CRC [149].
Autophagy has a complex and context-dependent role. On the one hand, it can protect against aberrant survival by promoting autophagic death of tumor cells, keeping homeostasis, and removing disfunctional organelles in early stages, whereas on the other hand, it can promote tumor proliferation by supporting immune evasion, epithelial-mesenchymal transition (EMT), angiogenesis, and resisting the therapeutic effects when cancer has advanced.
AKT: Ak strain transforming; AMPK: AMP-activated protein kinase ; ATG10: autophagy-related gene 10; EGFR: epidermal growth factor receptor; EMT: epithelial–mesenchymal transition; LC3: microtubule-associated protein light chain 3; Mapk14: mitogen-activated protein kinase 14; MHC-I: major histocompatibility complex class-I; mTOR: mammalian target of rapamycin; NF-κB: nuclear factor kappa-B; PDL1: programmed death ligand 1; PI3K: phosphoinositide 3-kinase; ROS: reactive oxygen species; SIRT1: sirtuin 1; SOX2: SRY-related HMG-box transcription factor 2; SphK1: sphingosine kinase 1; SQSTM1: sequestosome 1; TCR: T cell receptor

1.3.1 Autophagy and TLR9 signaling in cancer

1.3.1.1 The role of TLRs in the regulation of autophagy

TLR-autophagy interactions generate innate immune responses [146]. The canonical type of TLRs can induce autophagy, according to recent studies. In addition, several TLRs induce LAP in macrophages, dendritic cells, and neutrophils. This suggests these pathways aid cellular defense [143,146,151,152]. Additionally, TLRs may intrinsically initiate autophagy. Phagocytosis is the main defensive mechanism of innate immunity. TLR signaling in macrophages activates transduction pathways that link the autophagic pathway to phagocytosis. **Figure 10** shows that autophagy can also affect TLR signaling [143,152,163].

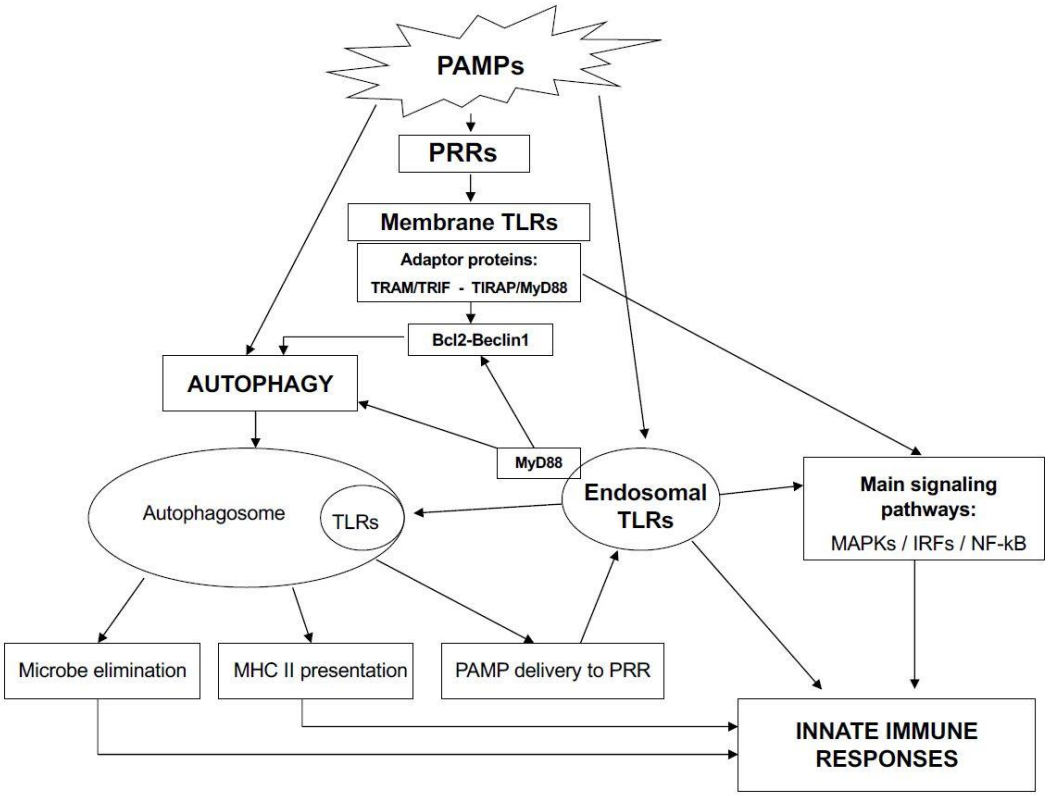

FIGURE 10 | Schematic illustration regarding the relation of PAMPs to (PRR) TLRs and autophagy in respect of signaling and regulatory loops [158].
PRR/TLR-ligands engage different adaptor proteins to initiate signaling. The association of *Bcl2 and Beclin-1* with *MyD88* represents a basic molecular mechanism in linking TLR and autophagy signaling. Autophagy now is regarded as a recent output of TLR signaling deeply affecting innate immunity.
Bcl-2: B-cell lymphoma 2; IRF: interferon regulatory factor; MAPK: mitogen activated phosphokinase; MHC II: major histocompatibility complex class II; MyD88: myeloid differentiation primary response gene; NF-kB: nuclear factor-κB; PAMP: pathogen-associated molecular pattern; PRR: pattern recognition receptors; TIRAP: Toll-interleukin 1 receptor domain-containing adapter protein; TRAM: Toll-like receptor 4 adaptor protein; TLR: Toll-like receptor; TRIF: Toll-interleukin 1 receptor-domain-containing adapter-inducing interferon

However, the activation of TLR7 has not been shown to induce pDC autophagy [154]. Other immune system triggers do not induce autophagy in DCs, which naturally have strong autophagy. Instead, other signaling pathways may prevent autophagy [153]. TLRs initiate NF-κB/MAPK (*ERK, p38, JNK*) and IRF3/7 signaling pathways [155]. *MyD88* and TRIF are the main adaptor proteins that activate autophagy after TLR activation [147,155,156]. After the TLR signaling pathway is activated, *Beclin-1* also joins with *MyD88* and TRIF, which separates it from the *Bcl-2* binding complex. Additionally, TRAF6 ubiquitination of *Beclin-1* increased TLR4-induced autophagy. In contrast, the deubiquitinating enzyme A20 had the opposite effect. The activation of *NF-κB* in response to TLR stimulation may hinder autophagy regulation [155-157].

1.3.1.2 The role of autophagy in the regulation of TLRs

Autophagy protects cells from stress; thus, its role in regulating TLR-mediated pro-inflammatory responses is not surprising [159]. Inflammation is a major inhibitor of autophagy [152]. It directly affects inflammation by inhibiting adaptor proteins MyD88 and TRIF as well as killing invading microorganisms [160,161]. Aggregating TLR adaptors can generate large cytoplasmic aggregates. Autophagy mostly inhibits *TLR* signaling, which is important. This effect may be reversible in pDCs. [159]. Several autophagy proteins inhibit TLR-mediated signaling. In response to LPS-induced TLR4 stimulation, *ATG16L1*-deficient macrophages generate IL-1 and IL-18 due to increased caspase-1 activation [161]. In addition, *LC3B* or *Beclin-1* deficits impair macrophage autophagy, causing mitochondrial dysfunction. ROS production increased with accumulation [163].

In autophagosomes, autophagy sequesters endogenous viral or self-antigens to deliver them to MHC class II antigens. This method presents MHC II-restricted cytoplasmic antigens to T cells [163]. Contrary to common assumptions, autophagic machinery may transport PAMPs to endosomal TLRs, similar to antigen presentation. This suggests that autophagy enhances TLR-PAMP recognition and TLR-induced effects. **Figure 11** shows that autophagy may initiate an innate immune response before TLR activation [155].

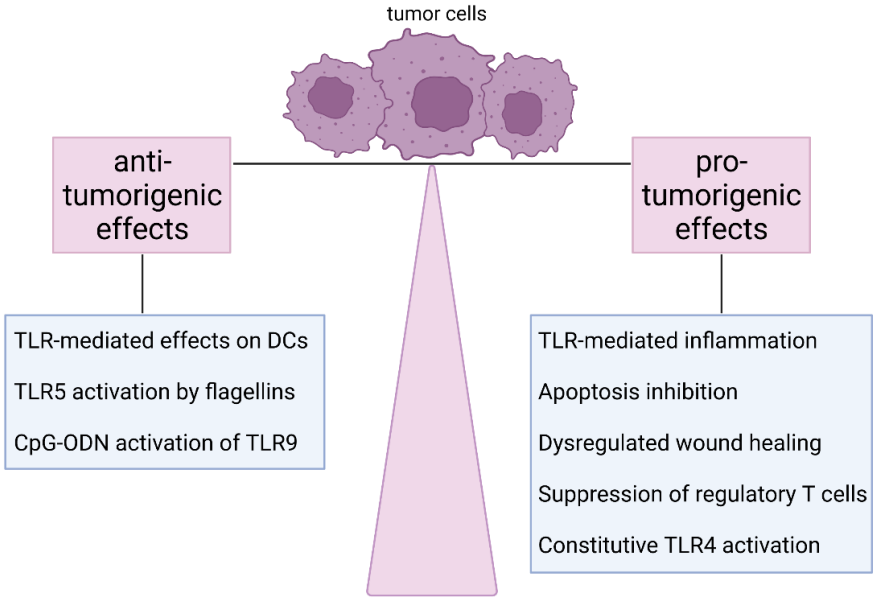

FIGURE 11 | The dual-faced role of TLR signaling in carcinogenesis [158].
While some direct and indirect actions of TLR-signaling act largely as an anti-tumorigenic factor, other effects may promote cancer development. (Created with BioRender.com)
TLR: Toll-like receptor; DC: dendritic cell; CpG-ODN: CpG-oligodeoxynucleotide

1.3.1.3 The role of TLRs and autophagy in cancer

The relationship between *TLR* and autophagy signaling in cancer cells is poorly understood. LPS and polyinosinine:polycytidylic acid (poly(I:C)) interact with TLR4 and TLR3 to activate autophagy in lung cancer cells, which increases the amount of cytokines and chemokines. Promoting *TRAF6* ubiquitination enhances cancer cell invasion and migration. The adaptor TRIF induces autophagy. However, autophagy suppression significantly inhibited *MAPK* and *NF-κB* signaling pathways. These pathways' activation depends on TLR3 and TLR4. Thus, this alteration may be a viable lung cancer treatment [164].

In controlled experiments, GO activated TLRs and induced autophagy in CT26 colon cancer cells. Cancer cells ingested GO, stimulating autophagy and TLR4 and TLR9 activation. The *MyD88* and *TRAF6* adaptors were shown to regulate GO-induced autophagy. In mice, GO boosted autophagy, cellular death, and malignant cell immune responses while suppressing tumor development [165].

Many cancer cells express TLR9, which is activated by the identification of unmethylated CpG-ODNs, a subgroup of DAMPs. Proteomics analysis of tumor cells has

found that bacterial CpG patterns affect several proteins, including autophagic ones [166]. Many parallels exist between autophagy and the CpG-TLR9 pathway. The study found that colon, breast, and prostate tumor cell lines promote autophagy. By showing that this induction is TLR9-dependent, TLRs and cancer autophagy are linked [167]. Autophagy might cause cells to die and help the MHC II pathway present endogenous cytosolic proteins. Thus, bacterial CpG patterns may stimulate tumor antigen presentation in cancer, boosting the immune response. The data above provide unique insights into how bacterial CpG patterns affect TLR9-expressing tumor cells, revealing a new therapeutic approach [168].

1.4 cfDNA in tumors

Tumor-associated cfDNA's liquid biopsy capability makes it appealing for several therapeutic uses [169,170]. Despite its limitations, surgical biopsy/histology is the main cancer diagnostic method. This includes invasive and transient static cancers [171]. However, tumor cfDNA detection allows dynamic cancer development monitoring and genetic variety insights [172-174]. Recent genetic studies of plasma and tissue samples have shown considerable agreement, spurring more therapeutic research [175-178]. Early tumor cfDNA detection is a common method in various cancer types [179]. But cfDNA purification and handling are unstandardized. Centrifuges, purification kits, and blood collection tubes can impact cfDNA yield and analysis [180,181]. Therefore, more sensitive and reproducible procedures are needed. Screening using tumor cfDNA and conventional markers looks ideal [182-184]. Several studies have demonstrated that cfDNA can detect minor residual sickness after surgery or therapy in various cancer types. cfDNA is predictive because it predicts disease recurrence [185-187]. Tumor cfDNA genotyping is used in oncology for a variety of purposes. It helps choose the optimal treatment, track results, and uncover genetic factors that promote cancer and medication resistance. Clinical use of tumor cfDNA is imminent [188].

Chronic inflammation has been a hallmark of cancer since Colotta [189], Hanahan, and Weinberg [190,191] pioneered it. Numerous investigations have shown that people with various neoplastic diseases have higher cfDNA levels. One characteristic of cfDNA is its ability to cause inflammation. It is reasonable to explore cfDNA-induced inflammation's carcinogenic implications [192].

TLR9 cfDNA detection produces both positive and negative effects on tumor cells. CRC tissues overexpress TLR9. Through the *TLR9-MyD88* signaling pathway, cfDNA from colon cancer cells or the TLR9 agonist CpG-ODN2395 increased CRC cell growth, migration, invasion, and IL-8 production [192]. Findings show breast cancer secretes cfDNA primarily actively. Activating the *TLR9-NF-κB-cyclin D1* pathway with cfDNA can boost hormone-receptor-positive breast cancer cell proliferation [193]. The host's TLR9 recognizes tumor cfDNA to modulate the immune response to malignancies following chemotherapy, according to previous research. TLR9 helps DCs mature and migrate from the tumor microenvironment to nearby lymph nodes. These DCs stimulate lymph node tumor-specific cytotoxic T lymphocytes, generating powerful anti-tumor responses [194]. The processes described above are illustrated in **Figure 12**.

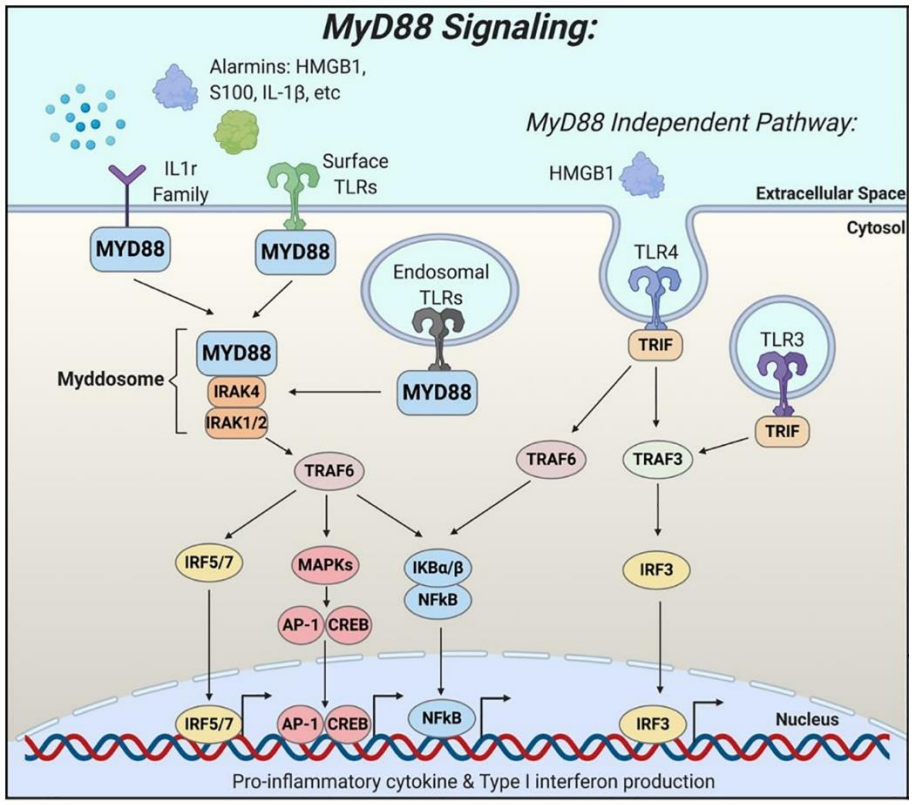

FIGURE 12 | MyD88 signaling pathways [195].
TLRs and cytokine receptors recognize alarmins, which activate MyD88 and trigger an immune response, in addition to cell-specific functions.
AP-1: activator protein 1; CREB: cAMP-response element binding protein; HMGB1: high mobility group box 1; IL-1r: interleukin-1 receptor; IκBα/β: kinase complex (IKK); IRAK1/2/4: interleukin receptor-associated kinases 1/2/4; IRF3/5/7: interferon regulatory factor3/5/7; MAPK: mitogen activated phosphokinase/protein kinase; MyD88: myeloid differentiation primary response gene 88; NF-κB: nuclear factor kappa-B; TLRs: Toll-like receptors; TRAF6: tumor necrosis factor receptor-associated factor 6; TRIF: Toll-interleukin-1 receptor-domain-containing adapter-inducing interferon; S100: small acidic protein 100

In normal mitosis, the nucleosome competes with dsDNA to prevent cGAS activation. Thus, *cGAS-STING* signaling is incomplete. IRF3 phosphorylation and accumulation during mitotic arrest result from reduced *cGAS-STING* signaling. This reduces inflammation without increasing type 1 IFN. It induces apoptosis [196]. The cancer medicines taxol, paclitaxel, and taxane function in this manner. In some tumor types, overexpression of *cGAS-STING* reduces the infiltration of inflammatory immune cells, which worsens the prognosis [196,197]. Patients with lung cancer who had *cGAS* downregulation experienced a higher death rate [198]. Further research suggests that the *cGAS-STING* signaling pathway controls the immunological milieu of several tumor microenvironments. *STING* signaling pathway activation enhances protective benefits of immunotherapy and robust tumoricidal T-cell immune response [199,200]. NEAT1 suppresses the *cGAS-STING* signaling pathway and limits cytotoxic T cell entry into the tumor microenvironment in mice, encouraging tumor development [201].

AIM2 has anti-cancer benefits independent of inflammasome activity [62,202]. AIM2 includes colitis-associated cancer, hereditary nonpolyposis colorectal cancer, and cutaneous squamous cell carcinoma that have been chemically started [202-204]. AIM2 promotes non-small-cell lung cancer tumor development by altering mitochondrial dynamics [205,206]. AIM2 promotes oral squamous cell carcinoma, benign prostatic hyperplasia, and chemically-induced hepatic cell carcinoma [207–209]. Furthermore, AIM2 has been suggested to slow down the progression of HCC [210].

Genometastasis [211] is a popular theory that may explain metastasis-development experimental differences [212]. Cancer cfDNA with oncogene pieces can act like oncoviruses, providing an alternative metastatic route. Secretomes and horizontal DNA transfer between *in vitro* cells and species support the idea [213-216].

To demonstrate genometastasis, the cfDNA from human CRC was analyzed for *KRAS*, *p53*, and *HBB* gene mutant pieces. NIH-3T3 mouse tumor cells without the mutant gene pattern were subcutaneously implanted into NOD-SCID mice after 20 days of cfDNA incubation. Aggressive "transformed" mouse tumor cells have mutant *KRAS* genes. In another study, tumorous cfDNA in human adipose tissue stem cells did not cause gene alterations or cancer [212]. Cancer-derived cfDNA is implicated in malignant transformation in cell culture and animals [214,215,217,218].

Tumor development and metastasis are linked to increased NET production [219]. Granulocytes release DNA that binds and kills pathogens in the extracellular environment, synthesizing NET. This boosts cell adhesion, invasion, and immune system evasion [220]. DNA immobilizes and supports CCDC25 receptors. HMGB1, neutrophil elastase, ROS, and the TLR4-TLR9 pathway activate tumor cells [221].

However, evidence reveals tumor tissue NETs may be hazardous. NETs inhibit cancer cell proliferation by inducing apoptosis in Caco-2 and AML cells and suppressing melanoma cell migration and survival [222,223]. In a CT-26 mouse model of colorectal adenocarcinoma, oncolytic VSV caused neutrophil-dependent microvessel clot formation in tumor vessels. [224]. **Table 1** lists putative cfDNA effects on tumorgenesis.

TABLE 1 | The possible effects of cfDNA on tumor formation [86]

Harmful and beneficial impacts of cell-free DNA in tumors

Protumor effects			Anti-tumor effects
TLR9-MyD88 + ODN2395	boosts cell growth, migration, invasion, and IL8 secretion [206]	**cfDNA sensing by TLR9**	modulates anti-tumor immunity in response to chemotherapy [208]
TLR9-NF-kB-Cyclin D1	stimulation of cell proliferation [207]		promotes maturation and migration of DCs to lymph nodes [208]
cGAS-STING overexpression	reduces intratumoral inflammatory cell infiltration [210]		activates tumor-specific cytotoxic T cells [208]
	leads to poor prognosis [210]	**low expression of cGAS-STING**	ameliorates inflammation [209]
cGAS downregulation	increases mortality [211]		enhances apoptosis [209]
cGAS-STING inhibition by NEAT1	promotes tumor growth [214]	**STING activation**	improves the protective effects of immunotherapy [212]
AIM2 cfDNA sensing	modifies mitochondrial dynamics [218,219]		enhances T cell-mediated anti-tumor immunity [213]
cfDNA containing secretome	favors to supportive peritumoral milieu[229]	**AIM2 (regardless of inflammasome activation)**	favors tumor cell survival [100,215-217]
horizontal DNA transfer	favors to supportive peritumoral milieu [227,228,230,231]	**NET deposition**	displays cytotoxic effects [235,236]
NET formation	enhances adhesion, invasion, immune escape [232]		inhibits cell growth, migration, survival [235,236]
	serves as a scaffold and trapping element [234]		induces apoptosis [235]
NET + TLR4-TLR9-HMBG1	activates neutrophils [234] activates tumor cells [234]		

1.5 HGFR: functions, relationship with autophagy and cancer

c-Met encodes the hepatocyte growth factor receptor (HGFR). Alpha and beta subunits of this transmembrane RTK protein are disulfide-bonded. In physiological circumstances, epithelial, muscle, hematopoietic, immunological, and neurological cells express HGFR. Many tumor and stromal cells express HGFR in cancer [225]. HGFR binds HGF. Fibroblasts and macrophages produce HGF, which has pleiotropic effects such as promoting cell survival, tissue preservation, regeneration, and anti-inflammatory effects [226]. HGF regulates cell motility, adhesion, and cytokine production [227].

There are two ways that HGFR can be activated: the canonical pathway involves other receptor dimerization, and the conventional pathway involves HGF binding and homodimerization [228]. Activation of HGFR promotes CRC malignancy by activating signaling pathways that influence cancer cell survival, proliferation, motility, migration, and invasion [229]. Metastasis through epithelial-to-mesenchymal transition necessitates signaling within and outside of this pathway [226]. By causing DNA double-strand breaks and perhaps lowering tumor hypoxia, HGFR inhibition made HT29 colorectal cancer cells more irradiable [230]. The HGF/HGFR system - as illustrated in **Figure 13** - can lead to tumor growth through transcriptional activation, gene amplification, mutation, or stimulation at the autocrine or paracrine level. Hepatocellular, pancreatic ductal, and colorectal malignancies activate HGF/HGFR [231]. This aberrant activation promotes the action of growth factors and oncogenic receptors, stimulating cell proliferation and metastasis [232]. Thus, HGF/HGFR inhibition is a promising targeted cancer therapy.

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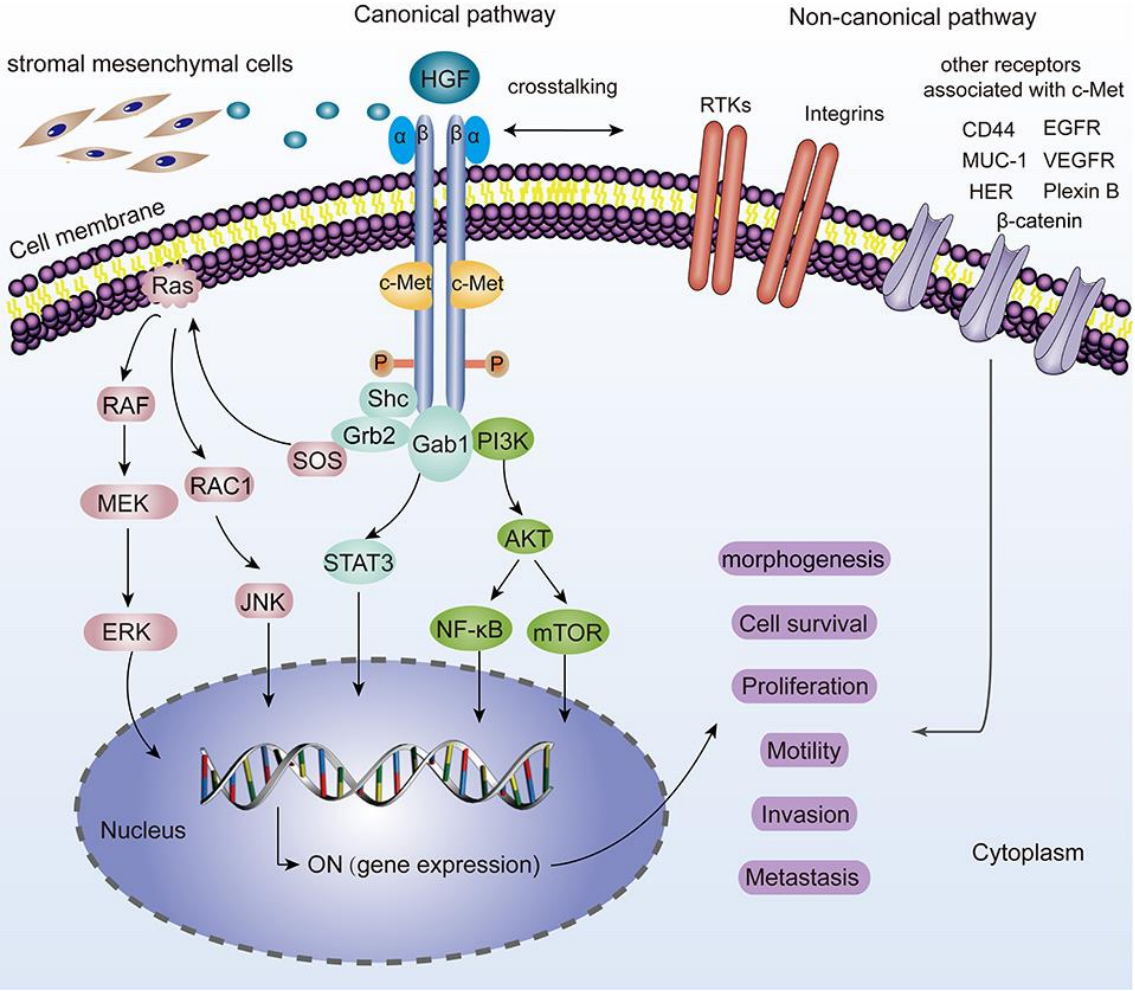

FIGURE 13 | The canonical and non-canonical pathways of HGF/c-Met (HGFR) [233].
The binding of HGF to c-Met results in the dimerization of two c-Met molecules, which triggers the autophosphorylation of tyrosine residues and activates many downstream signaling pathways, including MAPK/ERK, STAT3, and PI3K/AKT signaling. Phosphorylation of JNK also activates a variety of downstream substrates, including transcription factors like AP-1 and apoptosis-related Bcl-2, Bax, among others. All of these basically drive a plethora of cell phenotypes such as morphogenesis, survival, proliferation, motility, invasion, and metastasis. "ON" signifies the activation of gene expression. Other receptors, like EGFR, MUC-1, VEGFR, CD44, Plexin B1, HER, Integrin 64, β-catenin, and others, bind to c-Met and activate it. This leads to the activation of the non-canonical pathways.
AKT: Ak strain transforming; CD44: cluster of differentiation 44; c-Met: C mesenchymal epithelial transition factor; EGFR: epidermal growth factor receptor; ERK: extracellular signal-regulated kinase; Gab1: GRB2 associated binding protein 1; Grb2: growth factor receptor-bound protein 2; HER: human epidermal growth factor receptor; HGF: hepatocyte growth factor; JNK: C-jun N-terminal kinase; MEK: mitogen-activated extracellular signal-regulated kinase; mTOR: mammalian target of rapamycin; MUC-1: transmembrane glycoprotein mucin 1; NF-κB: nuclear factor kappa-B; P: phosphor; PI3K: phosphoinositide 3-kinase; RAC1: Ras-related C3 botulinum toxin substrate 1; RAF: rapidly accelerated fibrosarcoma; Ras: GTPase protein; RTKs: receptor tyrosine kinases; Shc: adaptor protein; STAT3: signal transducer and activator of transcription 3; SOS: sinusoidal obstruction syndrome; VEGFR: vascular endothelial growth factor receptor

Anti-HGF and anti-HGFR antibodies, as well as ATP-competitive and ATP-non-competitive small-molecule c-Met inhibitors [234,235]. It has been shown that the HGFR-EGFR interaction causes cancer [236]. Instead of being intrinsic, metastatic colorectal cancer acquires HGFR amplification after EGFR inhibition [237].

Evolution has preserved proteolysis. To maintain cellular homeostasis, damaged cellular components and energy are eliminated and recycled [238,239]. In preclinical trials, protective autophagy inhibition has been used alongside chemotherapies or targeted treatments to boost their effectiveness in certain cancers [240]. HGFR inhibition causes autophagy activation and inhibition in cancer cells [241,242]. Recent investigations have shown that HGFR-mediated autophagy requires the *HGFR*/*mTOR/ULK1* cascade. Targeting autophagy with therapeutic treatments may help HGFR-tyrosine kinases combat Met-amplified cancer cells [237,240,243].

A recent study has shown that *HGFR* gene DNA methylation changes over time affect the HGF/HGFR signaling cascade [244]. Additionally, DNA aptamers have beneficial chemical characteristics that can be used to build growth factor mimetics, particularly HGFR-targeting ones [245,246]. The discovery of powerful HGF-targeting drugs is crucial to cancer therapy. Inhibitory DNA aptamers that target human HGF may treat certain cancers [247].

1.6 IGF1R: functions, relationship with autophagy and cancer

IGF1R is a transmembrane receptor tyrosine kinase, which consists of an alpha and beta subunit. Insulin, IGF-1, and IGF-2 bind to IGF1R. The IGF1R-β receptor phosphorylates IRS1/2, SHC, and 14-3-3 after ligand stimulation. Downstream signaling pathways include *PI3K/AKT*, *JAK/STAT*, *Src*, *FAK*, and *RAS/MAPK*. **Figure 14** shows how these pathways regulate apoptosis and cell development genes [249,250]. Normal physiological growth, development, and nutrition include IGF1R in many tissues [251].

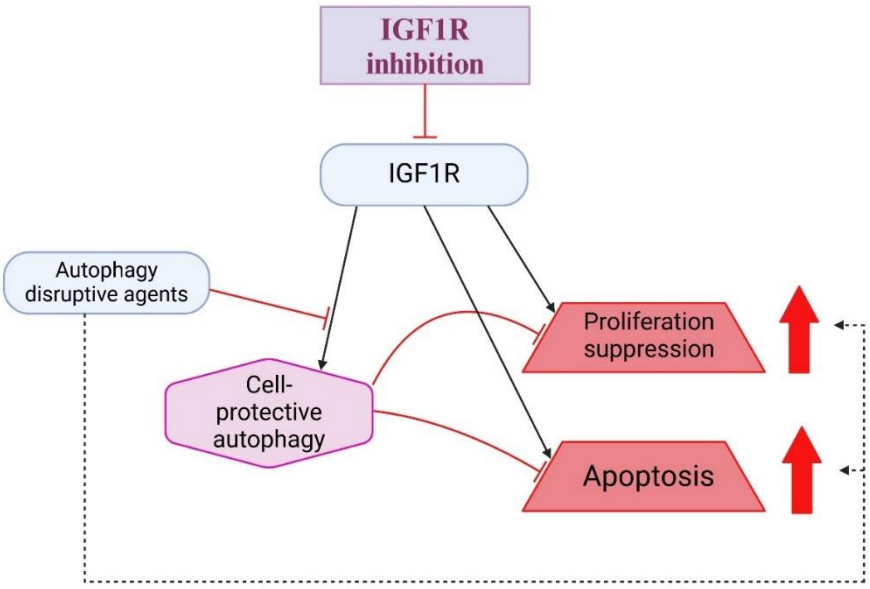

FIGURE 14 | Proposed model for the bi-directional IGF1R signaling-dependent modulation of the autophagic pathway [263].
IGF1R targeting via suppression of the "canonical" *PI3K/Akt/mTORC1* pathway stimulates the autophagy In case of IGF1R inhibition the simultaneously induced cell-protective autophagy could promote cell proliferation and suppress apoptosis, thus via autophagy antagonize its own original actions on cells. If IGF1R inhibition is combined with autophagy disruptive agents autophagy can be blocked, hence cancer cell proliferation will be suppressed and apoptosis enhanced. (Created with BioRender.com)
IGF1R: insulin-like growth factor receptor 1

IGF1R activation in malignancies promotes carcinogenesis, maintains the altered phenotype, progresses cancer development, increases cell migration, causes epithelial-mesenchymal transition, and imparts treatment resistance [252,253]. Normal tissues exhibited lower *IGF1R* gene and protein expression than malignant CRC tissues [254]. Higher levels of IGF1R are associated with worse CRC outcomes [255]. IGF1R is involved in tumor growth and progression; hence, decreasing it has helped several malignancies [256]. Preclinical studies have demonstrated that anti-IGF1R monoclonal antibodies and small-molecule inhibitors have substantial anti-tumor effects [256], but clinical trials in non-selected cancer patients have failed. This suggests tumor cells can bypass IGF1R inhibition [238].

A previous study has shown that self-DNA configuration, including methylation status and fragment length, greatly affects TLR9-mediated signaling pathways [119]. Insufficient evidence exists on *TLR* signaling and the *IGF1R* pathway. A recent study suggests that CpG-ODN, a TLR9 ligand, stimulates intestinal epithelial IGF1 synthesis

[257]. IGF1 also supports intestinal homeostasis by stimulating macrophage production, which suppresses the immune system [258].

In moderate and chronic colon inflammation, mRNA and protein levels of IGF1R in epithelial cells rise [259]. This may help inflammation-related genetic defects in epithelial cells proliferate and survive. In acute murine colitis, IGF1-stimulated macrophages produced IL-10 to reduce intestinal immunological inflammation [257]. Scientists debate the biological importance of the *IGF1/IGF1R* axis in colonic inflammation [259].

Insulin and cell surface receptors, particularly the insulin receptor and IGF1R, assist cancer cells in survival and growth [261]. High blood insulin levels can alter the IGF-IGF1R axis, a well-known cancer route [262].

There is an interconnection between the *IGF1R* signaling pathway and the autophagy process [263]. In addition, inhibiting or stimulating IGF1R in cancer cells has had different effects on autophagy [264-266]. Autophagy-disrupting drugs and IGF1R inhibitors can improve triple-negative breast cancer treatment, according to recent studies. Recent studies have found that targeting cancers related to IGF1 signaling with IGF1R inhibitor-based drugs is a promising treatment idea (**Figure 15**) [267]. In various cancer stages, targeting IGF1R may be helpful. However, IGF1R pharmacological alteration may have extra physiologic consequences, so be cautious. The current finding reveals that IGF1R suppression may impair mTORC2 function. Reduced mTORC2 function impacts PKC α and β activity. Thus, cytoskeleton alteration and endocytosis rate affect autophagosome formation. *IGF1R* suppression affects autophagy in both ways [266]. Pharmacological inhibition of an *IGF1R* pathway effector and increased autophagy may work together. The data also imply that dual mTORC1/2 catalytic inhibitors may limit autophagy over time. This inhibition may impair cancer cell viability [268-270].

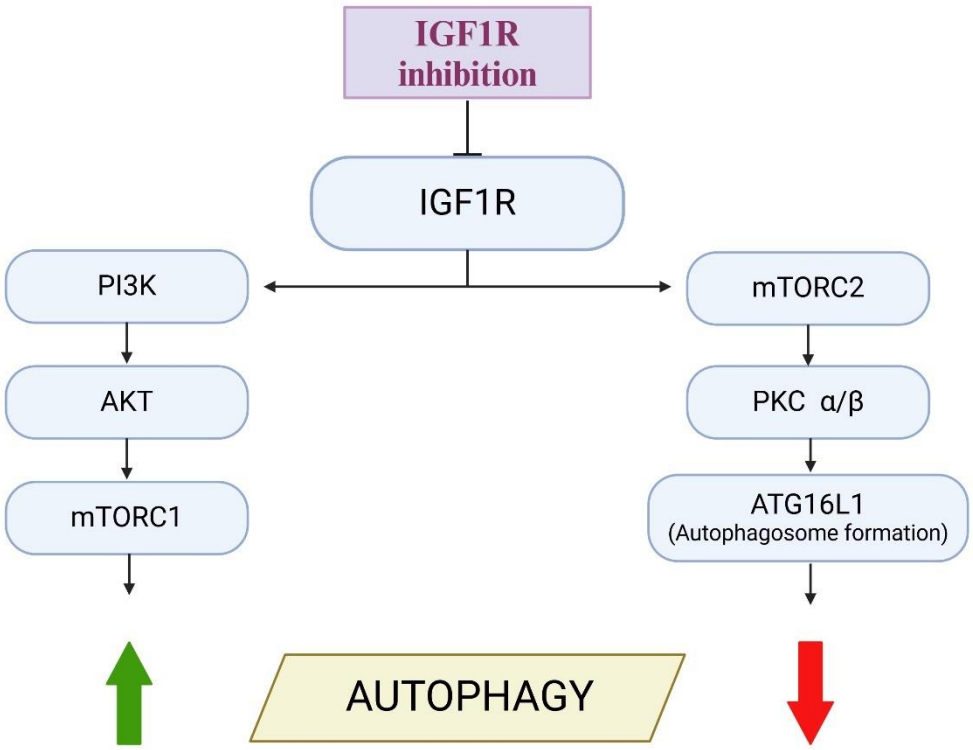

FIGURE 15 | Controversial therapeutic effects of IGF1R inhibition [263].
IGF1R targeting via suppression of the "canonical" *PI3K/Akt/mTORC1* pathway stimulates the autophagy process. However, it can also result in a reduced formation of autophagosomal precursors at the plasma membrane. IGF1R depletion inhibits mTORC2, which reduces the activity of PKC α and β. This finally negatively impacts autophagosome precursor formation. (Created with BioRender.com)
IGF1R:iInsulin-like growth factor receptor 1; PI3K: phosphatidylinositol-3-kinase; PKC: protein kinase C; AKT: Ak strain transforming; mTORC1/2: mammalian target of rapamycin complex 1/2; ATG16L1: autophagy-related protein 16-1

The link between autophagy and cell cycle progression is unclear. Earlier studies found that mitotic cells are more resistant to autophagy-inducing stimuli such as mTOR inhibition [271]. Recent research examines the chemopreventive properties of Boswellia serrata gum resin's active constituent, AKBA. AKBA's particular interaction with oncogenic proteins explains this focus [272,273]. Epigenetic modification by AKBA suppresses CRC cell proliferation [274]. A potent natural analog of AKBA (BA145) triggers dose- and time-dependent autophagy in pancreatic cancer cells [275]. BA145-induced autophagy halted the G2/M cell cycle and decelerated cell proliferation. BA145 induced autophagy by blocking *mTOR,* which activated *Akt* via *IGF1R/PI3K*. Akt feedback attenuated BA145-induced autophagy, cell cycle, arrest and cell death. This

suggests single-target cancer treatments are ineffective [275]. **Figure 16** illustrates the mechanisms.

Increasing data shows that regulating autophagy and suppressing IGF and the *IGF1R* system may improve insulin-associated inflammatory and neoplastic diseases in the colon. However, manipulating the IGF1R-autophagy process pharmacologically, whether alone or in combination, may have unforeseen pathobiological effects.

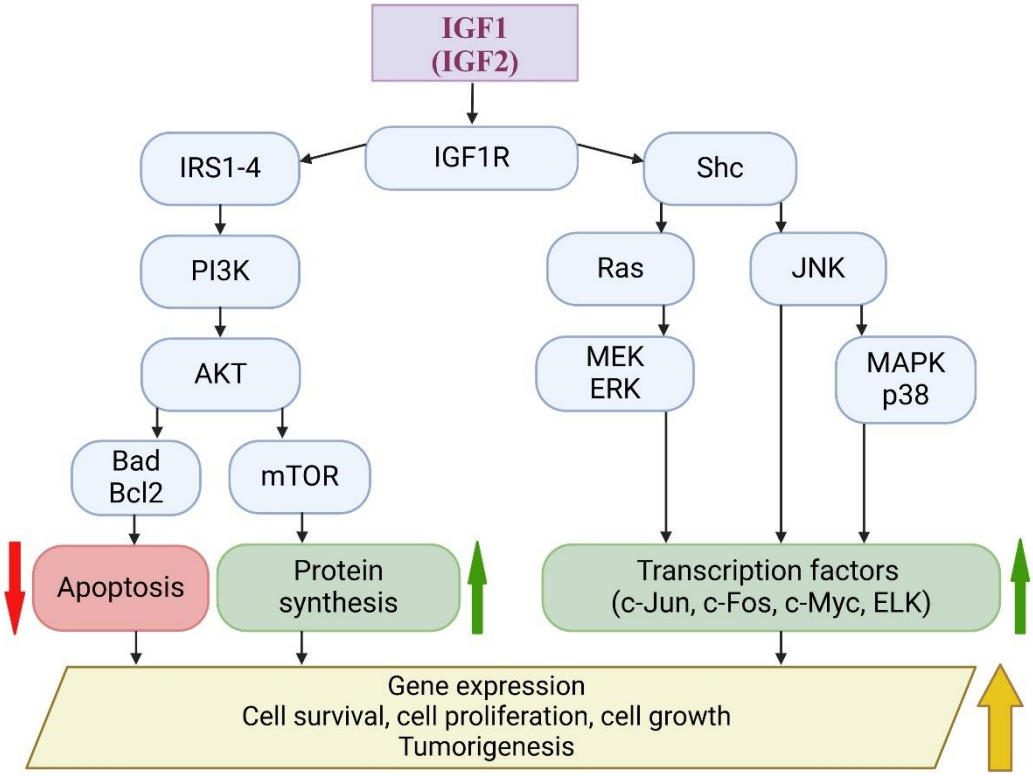

FIGURE 16 | The IGF/IGF1R axis: schematic representation of the composition and function [263]. Signaling of the IGF/IGF1R axis is mediated by IRS and Shc. *PI3K-AKT* activation is the predominant downstream event, but the *Ras/MEK/ERK* and *JNK/MAPK* pathways can also be activated. (Created with BioRender.com)

IGF: insulin-like growth factor; IGF1R: insulin-like growth factor receptor 1; IRS: insulin receptor substrate; PI3K: phosphatidylinositol-3-kinase; AKT: Ak strain transforming; mTOR: mammalian target of rapamycin; Bad: Bcl-2-associated death promoter; Bcl2: B-cell lymphoma 2; Shc: adaptor protein; Ras: GTPase protein; JNK: c-Jun N-terminal kinase; MEK: mitogen-activated extracellular signal-regulated kinase; ERK: extracellular regulated kinase; MAPK: mitogen-activated protein kinase; ELK: ETS domain-containing protein

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2. OBJECTIVES

1. To select a human colorectal cancer adenocarcinoma cell line that is suitable for the combined application of cfDNA-induced TLR9-mediated autophagy and HGFR/IGF1R inhibition.

2. To investigate the complex biological effects of TLR9-mediated autophagy and HGFR inhibition induced by cfDNA:
How do cfDNA treatments with different properties (i.e., genomic, fragmented, or hypermethylated) affect the metabolic activity, proliferation, autophagy response, and stem cell phenotype of the selected colorectal cancer adenocarcinoma cell line?

3. To investigate the complex biological effects of TLR9-mediated autophagy and IGF1R inhibition induced by cfDNA:
How does genomic cfDNA treatment affect the metabolic activity, proliferation, autophagy response, and stem cell phenotype of the selected colon cancer adenocarcinoma cell line?

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3. MATERIALS AND METHODS

3.1 Selection and maintenance of HT29 cell culture; self-DNA isolation

The selection of HT29 cells was made taking into account several aspects. There is basal TLR9 expression in HT29 cells, which is essential for induction with self-DNA [257]. Moreover, the *MyD88*-dependent and *MyD88*-independent *TLR* signaling pathways are intact in HT29 cells [277]. In HT29 cells, HGFR expression is high as compared to other CRC cell lines [278], and TLR and autophagy-mediated HGFR cross-activation is also present [167,279,280]. IGF1R expression in HT29 cells is moderate as compared to other CRC cell lines (e.g., SW480 or DLD-1) [281]. Also, in HT29 cells, elevated IGF2 expression can be detected, which is essential for both autocrine activation of IGF1R signaling and studying the effect of IGF1R inhibition [282]. HT29 cells adequately represent sporadic colon cancers [283].

Particular attention was paid to whether the inhibitors tested could cause proliferation inhibition in the given context. This graph (**Figure 17**) shows that in the DLD1 cell line, chloroquine treatment causes proliferation inhibition at concentrations as low as 10 µM, whereas in the HT29 cell line, proliferation inhibition occurs only at concentrations as high as 100 µM.

The application of inhibitors at given doses may not result in substantial suppression of cell growth. Beside HT29 cells, not all available colorectal cancer cell lines satisfy these criteria.

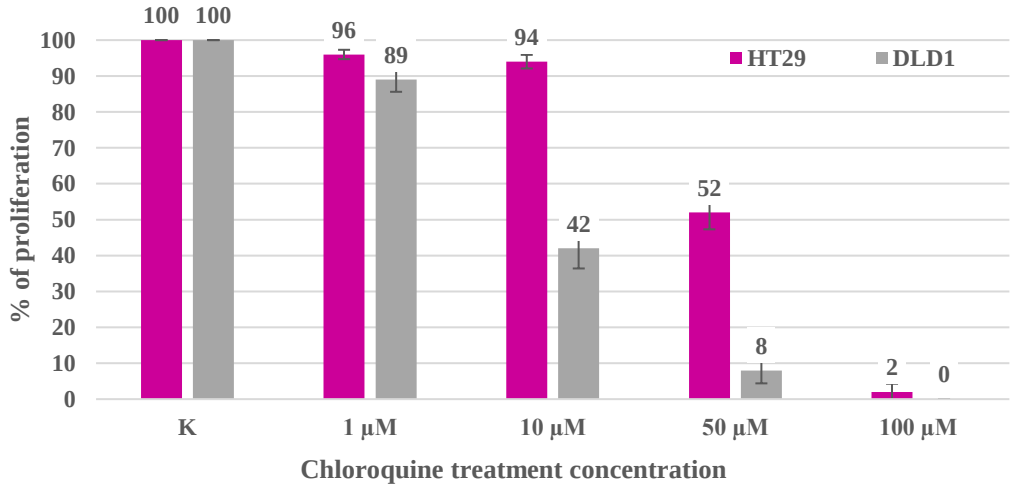

FIGURE 17 | Proliferation inhibition test on HT29 and DLD1 cell lines with chloroquine.

The HT29 undifferentiated colon adenocarcinoma cells were maintained in RPMI 1640 medium (Sigma-Aldrich, MO, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Standard Quality; PAA Laboratories GmbH, Austria), 125 μg/mL amphotericin B (Sigma-Aldrich, MO, USA), and 160 μg/mL gentamycin (Sandoz, Sandoz GmbH, Austria).

Genomic DNA was isolated from $5x10^7$ steady-state, proliferating HT29 cells. DNA isolation was performed by using High Pure PCR template preparation kit containing proteinase K (Roche GmbH, Germany). The DNA samples were treated with 5 μL RNase A/T1 Mix (Thermo Fisher Scientific, Germany). DNA concentration was determined by Nanodrop (Thermo Fisher Scientific, Germany). Gel electrophoresis determined that the fragment length of gDNA was approximately 10.000 base pairs [119]. According to the bisulfite sequencing analysis of *Ogoshi et al.* [284], the basal methylation status of HT29 cells' CpG sites is as follows:31.6 % in the low range; 11.6% in the middle; and 56.7% in the high range. According to the manufacturer's MALDI-TOF mass spectrometry measurements, the DNA samples were free of RNA, protein, or lipopolysaccharide contamination.

3.2 Fragmentation and hypermethylation of self-DNA

Genomic DNA was divided into three equal shares; the first one was neither fragmented nor hypermethylated (genomic DNA: gDNA). The second one was fragmented (fragmented DNA: fDNA) by ultrasonic fragmentation for 2 min. The third share was hypermethylated (methylated DNA: mDNA) using CpG methyltransferase (M.SssI) (New England Biolabs, Ipswich, USA). The length of the fragmented DNA shares was determined by agarose gel electrophoresis.

3.3 HT29 cell treatments

To incubate with the DNA samples, $5x10^5$ HT29 cells were seeded in a 12-well plate with RPMI 1640 supplemented with amphotericin B, gentamycin, and FBS, as previously described. After 24 hours, the medium was changed to RPMI 1640, supplemented with gentamycin but lacking FBS. Separate aliquots of 15 μg of modified self-DNA were dissolved in 200 μL of sterile phosphate buffered saline (PBS).

At 37°C, HT29 cells were incubated with the various DNA samples in a humidified atmosphere containing 5% CO_2 and 95% O_2. Only 200 μL of sterile PBS was added to the control cells. Cells were washed twice with 5 mL of sterile PBS and resuspended in a final volume of 5 mL of PBS after 72 hours.

3.4 Inhibition of TLR9, HGFR, IGF1R signaling and autophagy

For inhibition of TLR9-, HGFR-, IGF1R-signaling or autophagy, HT29 cells were pretreated with TLR9 antagonist (5 μM ODN2088; Invivogen, CA, USA), or 4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid (DISU; 4 μM; D3514 Sigma-Aldrich, Budapest, Hungary; diluted in dimethyl sulfoxide/DMSO; Sigma-Aldrich Budapest, Hungary/), or picropodophyllin (P) (0.05 μM; BML-EI372-0001; EnzoLifeSciences, BioMarker Ltd., Gödöllő, Hungary; diluted in dimethyl sulfoxide/DMSO; Sigma-Aldrich Budapest, Hungary/) or chloroquine (10 μM; C6628 Sigma-Aldrich, Budapest, Hungary; diluted in DMSO) for 1 hour before treatments with DNAs. All treatments were performed in triplicate. **Table 2** displays the treatment plan for HT29 cells in the HGFR experiments, while **Table 3** displays the IGF1R experiments**.**

TABLE 2 | Treatment plan for HT29 cancer cells in the HGFR experiments [248].
g/f/mDNA: genomic/fragmented/hypermethylated deoxyribonucleic acid; ODN2088: CpG oligonucleotide; DISU: 4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid

Sample groups	gDNA	fDNA	mDNA	ODN2088 ("O")	DISU ("D")	Chloroquine ("C")
K						
O				+		
D					+	
C						+
Kg	+					
Kf		+				
Km			+			
gO	+			+		
gD	+				+	
gC	+					+
gOD	+			+	+	
gDC	+				+	+
fO		+		+		
fD		+			+	
fC		+				+
fOD		+		+	+	
fDC		+			+	+
mO			+	+		
mD			+		+	
mC			+			+
mOD			+	+	+	
mDC			+		+	+

TABLE 3 | Treatment plan for HT29 cancer cells in the IGF1R experiments [276].
gDNA: genomic deoxyribonucleic acid; ODN: O, CpG oligonucleotide; K: non-treated, control; P: picropodophyllin; C: chloroquine; Kg: gDNA control; gO: gDNA + ODN2088; gP: gDNA + picropodophyllin; gC: gDNA + chloroquine; gOP: gDNA + ODN2088 + picropodophyllin; gPC: gDNA + picropodophyllin + chloroquine

Sample groups	gDNA	ODN2088 (”O”)	Picropodophyllin (”P”)	Chloroquine (”C”)
K				
O		+		
P			+	
C				+
Kg	+			
gO	+	+		
gP	+		+	
gC	+			+
gOP	+	+	+	
gPC	+		+	+

3.5 Cell viability and proliferation measurements

The use of the Alamar Blue assay served a dual purpose: partly to examine cell viability (metabolic activity) and partly to study cell proliferation [285].

The anti-proliferative effects of the 72-hour treatments were measured after a 4-hour incubation period using Alamar Blue (Thermo Fisher Scientific, Budapest, Hungary). The fluorescence was measured at 570–590 nm (Fluoroskan Ascent FL fluorimeter; Labsystems International Ltd., Budapest, Hungary), and the results were analyzed by Ascent Software.

As metabolic activity is not necessarily proportional to proliferative activity, manual cell counts (average cell numbers determined by using Bürker counting chambers) were also performed. Trypan Blue dye (302643 Sigma-Aldrich, Budapest, Hungary) was used to exclude dead cells.

3.6 Total mRNA isolation and NanoString analysis

Total mRNA from HT29 cells was extracted with the RNeasy Mini Kit (Qiagen, CA, USA) according to the prescription of the manufacturer. Quantitative (Nanodrop) and qualitative analysis (Bioanalyzer Pico 600 chip kit RNA program; RIN >8 in all cases) were performed.

To get the mRNA samples needed for gene expression tests on HT29 cells, the treated groups were multiplied three times. In HT29 samples, cell numbers ranged from 100,000 to 11,135,000 per well, and the recovered mRNA concentration ranged from 8 to 256 ng/μL per sample. mRNAs recovered from triplicates were pooled and used in the NanoString assay.

The custom mRNA Assay Evaluation panel (NA-SPRINT-CAR-1.0, nCounter SPRINT Cartridge) containing our custom gene code set (NA-XT-GXA-P1CS-04, nCounter GX Custom CodeSet) was designed by NanoString (the order was placed through Biomedica Hungaria Ltd., Budapest, Hungary). The NanoString experiments were carried out by RT-Europe Research Center Ltd. (Mosonmagyaróvár, Hungary; website: http://rt-europe.org/) as part of a contract work.

The selection criteria for examining the genes involved establishing an association between *c-Met/HGFR* or *IGF1R* and TLR9 signaling, apoptosis, cell proliferation, autophagy, and cancer cell stemness. **Supplementary Table 1** indicates the assayed genes with probe NSIDs.

3.7 Taqman real-time PCR analysis

For validating the NanoString gene expression analysis method, *mTOR* (ID: Hs00234508_m1), *ATG16L1* (ID: Hs01003142_m1), *LC3B* (ID: Hs00797944_s1), *BCN1* (ID: Hs01007018_m1), *HGFR* (ID: Hs01565584_m1), *IGF1R* (ID: Hs00609566_m1), *PI3KCA* (ID: Hs00907957_m1), *STAT3* (ID: Hs00374280_m1), *CD95* (ID: 4331182 Hs00236330_m1), and *TLR9* (ID: Hs00370913_s1) triplicated Taqman real-time polymerase chain reactions (PCRs) were used in an Applied Biosystems Micro Fluidic Card System. The measurements were performed using an ABI PRISM 7900HT Sequence Detection System as described in the user guide of products (http://www.appliedbiosystems.com, CA; United States). Gene expression levels for each individual sample were normalized to *GAPDH* (ID: Hs02786624_g1) expression. Mean relative gene expression was determined, and differences were calculated using the 2-ΔC(t) method. The whole cycle number was 45.

3.8 Immunocytochemistry for HGFR, IGF1R, CD133, TLR9 and autophagy

To detect *HGFR, IGF1R, CD133, TLR9,* and autophagy-associated *ATG16L2*, *Beclin-1,* and *LC3* protein expression, HT29 cell smears were incubated with rabbit polyclonal anti-Met culture supernatant antibody (1:100, Clone: C-12, Santa Cruz Biotechnology Inc.), anti-IGF1R monoclonal antibody (Chemicon International; Clone: 24-31; 1:50 dilution in PBS), anti-CD133/1-biotin antibody (1:100, Miltenyi, Germany), mouse anti-human monoclonal anti-TLR9 antibody (20 μg/mL; LS-B2341, clone: 26C593.2; LifeSpan BioSciences, WA, USA) and anti-ATG16L1-, anti-BECN1- and anti-MAP1LC3B antibodies (1:200, Antibody Verify, LA, USA) at 37°C for 1 hour. After three rounds of PBS rinsing, cell smears were treated for 40 minutes with an anti-rabbit EnVision polymerHRP conjugate kit (K4003, DAKO, Germany). Secondary immunodetection was performed according to the manufacturer's instructions using EnVision System Labeled Polymer-HRP K4001 (Anti-Mouse 1:1; DAKO, Germany). A Liquid DAB+ Substrate Chromogen System was used to convert the signal (DAKO, Germany). Smears of cells were then digitalized and analyzed using the CaseViewer software on a high-resolution PANNORAMIC 1000 FLASH DX instrument (3DHISTECH Ltd., Budapest, Hungary).

3.9 WES Simple and assessment of autophagic flux

The WES Simple (ProteinSimple 004-600, Minneapolis, MN, USA) method was also performed. A 12–230 kDa Separation Module (ProteinSimple SM-W004) was used for all the proteins (Phospho-mTOR (Ser2448) Rabbit Antibody /Cell Signaling; #2971; 1:50 dilution; 199 kDa/; mTOR (7C10) Rabbit mAb /Cell Signaling; #2983; 1:50; 200 kDa/; Anti-SQSTM1/p62 antibody [2C11] - BSA and Azide-free /Abcam; ab56416/; ATG16L1 (D6D5) Rabbit mAb /Cell Signaling; #8089; 1:50; 66-68 kDa/; Beclin-1 (D40C5) Rabbit mAb /Cell Signaling; #3495; 1:50; 60 kDa/; LC3B (D11) XP Rabbit mAb /Cell Signaling; #3868; 1:50; 14-16kDa/; Anti-β-actin (AC-74) Mouse mAb /Sigma Aldrich; A2228; 1:50; 48 kDa/; GAPDH (14C10) Rabbit mAb /Cell Signaling; #2118/) and either the Anti-Rabbit Detection Kit (ProteinSimple DM-001) or Anti-Mouse Detection Kit (ProteinSimple DM-002) was used, depending on the primary antibodies.

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3.10 Cell counting and interpretation of immunoreactions

At 200x magnification, 10 fields of view and at least 100 cells (mainly 110 cells) per field of view were examined in a semiquantitative manner in each digitalized sample. The percentage of immunopositive and non-immunoreactive HT29 cells was determined.
In the case of the TLR9 and HGFR immune responses, weak, moderate, and strong membrane staining and perinuclear cytoplasm staining were examined. In the case of the IGF1R immune response, weak, moderate, and strong membrane staining and perinuclear cytoplasm staining were examined. As for autophagy, weak, moderate, and strong *ATG16L1* and *Beclin-1* homogenous or spotted immunoreactions were detected in the cytoplasm. In the case of *LC3*, weak, moderate, and strong punctuated or spotted cytoplasmic immunoreactions were observed.

3.11 Transmission electron microscopy for evaluation of autophagy

For 60 minutes, HT29 cells in the wells were fixed in 2% glutaraldehyde (0.1M Millonig buffer, pH 7.4). The samples were post-fixed for 60 minutes at 4°C in the dark with 1% osmium tetroxide in 0.1 M sodium-cacodylate buffer. Cells were centrifuged and embedded in 10% gelatin in PBS (pH 7.4). The samples were embedded in Poly/Bed epoxy resin. Contrast staining of ultrathin sections (70-80 nm) with uranyl acetate and lead citrate, respectively. The JEM-1200EXII Transmission Electron Microscope was used to conduct ultrastructural analyses (JEOL, Akishima, Tokyo, Japan). In five HT29 cells per sample, the average number of autophagic vacuoles was counted (mean ± SD/cell).

3.12 Semithin sections

From the HT29 cell blocks fixed for TEM semithin sections were cut for viewing by digital microscope. The sections were stained with toluidine blue (toluidine blue O 4 g, pyronin 1 g, and borax 5 g in distilled water). The average number of proliferative cells was counted in five fields of view per sample (mean ± SD/sample).

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3.13 Statistical analysis

At least three independent experiments were conducted. Data on cell viability, cell number, and proliferation were presented as means ± SD. The $\chi 2$-test and Student's t-test were used for statistical analyses. $p < 0.05$ was considered statistically significant. In the case of immunocytochemistry, statistical analysis with one-way ANOVA and the Tukey HSD test was performed using R Core Team R version 3.5.3 (2019) [286].

Regarding NanoString gene expression analysis, after importing RCC files to the nSolver Analysis Software, quality checking was performed. Then agglomerative cluster heat maps were created. The Euclidean distance metric was used to calculate the distance between two samples (or genes) as the square root of the sum of squared differences in their log count values. The average linkage method was used to calculate the distance between two clusters. In the case of the WES Simple, the area of the tested proteins was multiplied by the values of the β-actin area for graphical representation.

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4. RESULTS

4.1 Cell viability and proliferation measurements (HGFR studies)

Treatment of HT29 cells with gDNA alone, ODN2088, DISU, chloroquine, or all three together increased their metabolic activity. However, cell viability was significantly reduced when TLR9 or autophagy inhibitor treatments combined with gDNA were also combined with DISU.

gDNA administration, as opposed to metabolic activity, inhibited the proliferation of HT29 cells. The co-treatment of ODN2088 and DISU significantly reduced the inhibitory effect of gDNA on cell proliferation. When treated alone, ODN2088 or DISU in combination with gDNA reduced the inhibitory effect of gDNA on cell proliferation. However, when combined, these treatments showed significantly enhanced efficacy. The co-treatment of gDNA, DISU, and chloroquine demonstrated the most efficacious suppression of HT29 cell proliferation, accompanied by elevated metabolic activity.

In isolation, fDNA treatment marginally enhanced cell viability; however, when combined with a TLR9 inhibitor, it substantially augmented metabolic activity; furthermore, it exhibited a moderate increase when combined with chloroquine or DISU. However, in the case of fDNA/ODN2088 and fDNA/chloroquine combinations, the metabolic activity of HT29 cells was reduced by DISU to the same extent as in the fDNA control samples.

The fDNA control samples produced a marginal increase in HT29 cell proliferation, which subsequently declined to varying degrees upon combination with both treatments. After DISU administration, the decrease in cell proliferation remained largely unchanged in the fDNA/ODN2088 combination. However, DISU in combination with fDNA and chloroquine had a minor effect on HT29 cell proliferation.

mDNA exhibited the greatest increase in cellular metabolic activity among all DNA varieties. In comparison to the mDNA control, the level of metabolic activity exhibited no change (DISU, ODN2088/DISU) or increased (ODN2088, chloroquine) throughout the interventions; it only decreased significantly when DISU and chloroquine were administered concurrently.

A marginal reduction in cell proliferation was observed in response to mDNA treatment; a decline was observed in response to all treatments, most notably autophagy

inhibition. With mDNA/DISU or mDNA/ODN2088/DISU regimens, however, a substantial increase in cell proliferation was observed. Notably, when mDNA, ODN2088, and DISU were co-administered, there was a concurrent increase in cell proliferation and a substantial reduction in metabolic activity. The data pertaining to cell proliferation, viability, and cell count are presented in **Table 4** and **Figure 18**.

TABLE 4 | Numerical data of metabolic activity, cell number and proliferation (HGFR studies) [248].
*represents significant alteration as compared to K (control), non-treated sample ($p<0.05$; n=3).
g/f/mDNA: genomic/fragmented/hypermethylated deoxyribonucleic acid; O: ODN2088 CpG oligonucleotide; D: DISU; C: chloroquine; SD: standard deviation

Sample	Metabloic activity mean % (± SD)	Average cell number (± SD)	Proliferation % (± SD)
K	100 ± 1.1	800,000 ± 8,800	100 ± 1.1
O	120.17 ± 4.5*	760,000 ± 32,680	95 ± 4.3
D	111.41 ± 3.8	810,000 ± 25,920	101.25 ± 3.2
C	116.23 ± 2.9*	775,000 ± 31,775	96.87 ± 4.1
Kg	127.51 ± 3.1*	220,000 ± 9,900	27.5 ± 4.5*
Kf	112.61 ± 2.2	855,000 ± 31,635	106.87 ± 3.7
Km	147.87 ± 3.4*	720,000 ± 19,440	90 ± 2.7*
gO	139 ± 3.1*	270,000 ± 3,780	33.75 ± 1.4*
gD	134.44 ± 2.7*	310,000 ± 6,510	38.75 ± 2.1*
gC	123.55 ± 3.1*	230,000 ± 4,370	28.75 ± 1.9*
gOD	75.75 ± 2.6*	690,000 ± 24,840	86.25 ± 3.6*
gDC	90.99 ± 3.3	100,000 ± 1,600	12.5 ± 1.6*
fO	198.02 ± 4.7*	745,000 ± 23,840	93.12 ± 3.2
fD	120.87 ± 3.7*	665,000 ± 21,945	83.12 ± 3.3*
fC	121.18 ± 2.5*	560,000 ± 13,440	70 ± 2.4
fOD	99.61 ± 3.7	730,000 ± 26,280	91.25 ± 3.6
fDC	107.62 ± 3.2	640,000 ± 17,920	80 ± 2.8*
mO	155.15 ± 4.1*	740,000 ± 25,160	92.5 ± 3.4
mD	141.85 ± 3.9*	875,000 ± 36,750	109.37 ± 4.2
mC	183.48 ± 4.6*	560,000 ± 16,240	70 ± 2.9*
mOD	90.12 ± 2.5	1,140,000 ± 60,420	142.5 ± 5.3*
mDC	92.34 ± 3.1	580,000 ± 9,860	72.5 ± 1.7*

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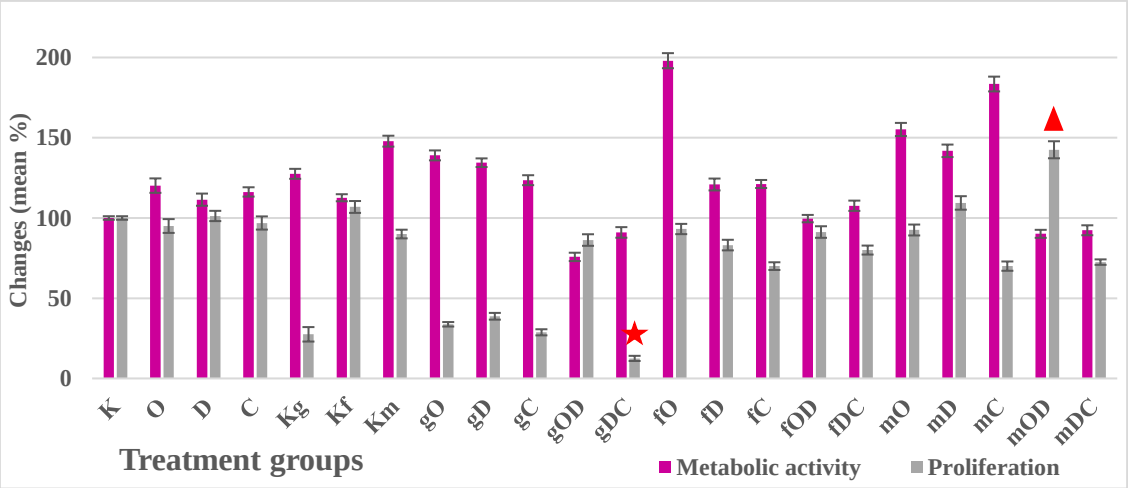

FIGURE 18 | Changes in the metabolic activity (magenta) and proliferation (grey) of the studied cell groups under the influence of each treatment combination in HGFR studies [248].
The red star indicates the lowest (group gDC), while the red triangle indicates the highest proliferative activity (group mOD).
g/f/m: genomic/fragmented/hypermethylated DNA; K: control; O: ODN2088 CpG oligonucleotide; D: DISU; C: chloroquine

4.2 Cell viability and proliferation measurements (IGF1R studies)

The metabolic activity of the HT29 cells was significantly ($p<0.05$) increased in all treatment groups except the gOP combination as compared to K (control, non-treated cells). The P treatment exhibited the highest metabolic activity.

The Kg treatment group of cells had significantly lower (p 0.05) cell proliferation compared to K.

When gDNA, ODN2088, picropodophyllin, and chloroquine were co-treated (i.e., gO, gP, gC), effective inhibition of HT29 cell proliferation with high metabolic activity was observed. The combination of gDNA, ODN2088, and picropodophyllin (i.e., gOP) raised proliferative activity back to levels close to those of the non-treated control group.

Viability, cell number, and proliferation data are illustrated in **Table 5** and **Figure 19.**

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TABLE 5 | Numerical data of metabolic activity, cell number and proliferation (IGF1R studies) [276].
*represents significant alteration as compared to K (control), non-treated sample ($p<0.05$; n=3).
g: genomic DNA; O: ODN2088 CpG oligonucleotide; P: picropodophyllin; C: chloroquine; SD: standard deviation

Sample	Metabloic activity mean % (± SD)	Average cell number (± SD)	Proliferation % (± SD)
K	100 ± 1.1	800,000 ± 8,800	100 ± 1.1
O	120.17 ± 4.5	760,000 ± 32,680	95 ± 4.3
P	142.15 ± 4.7*	810,000 ± 25,920	101.25 ± 1.8
C	116.23 ± 2.9	775,000 ± 30,775	96.87 ± 4.1
Kg	127.51 ± 3.1	220,000 ± 9,900	27.5 ± 4.5*
gO	139 ± 3.1*	270,000 ± 3,780	33.75 ± 1.4*
gP	119.57 ± 3.2	270,000 ± 7,020	33.75 ± 2.6*
gC	123.55 ± 3.1	230,000 ± 4,370	28.75 ± 1.9*
gOP	91.3 ± 2.4	660,000 ± 16,500	82.5 ± 2.5
gPC	127.38 ± 2.8	250,000 ± 9,250	31.25 ± 3.7*

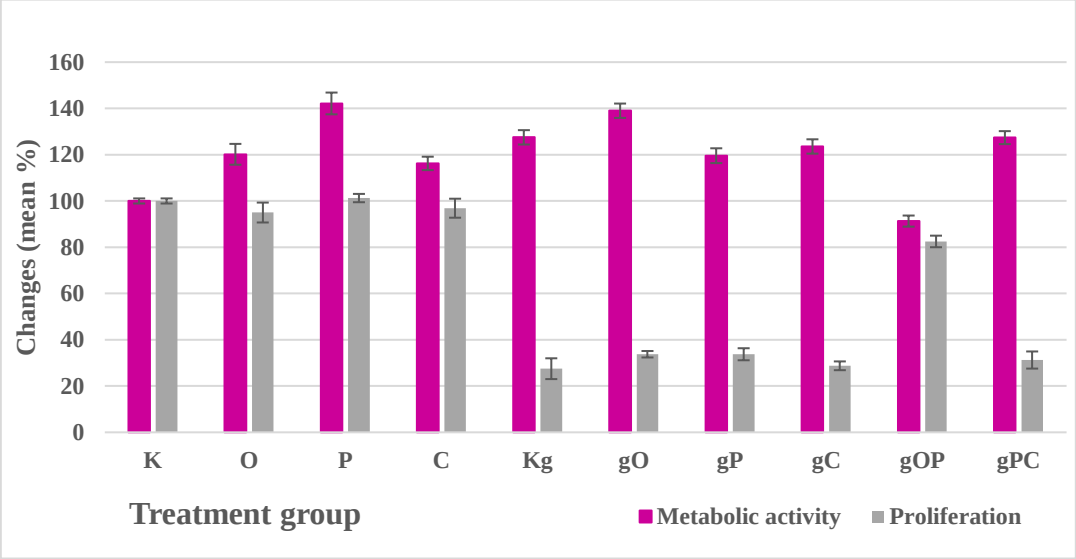

FIGURE 19 | Changes in the metabolic activity (magenta) and proliferation (grey) of the studied cell groups under the influence of each treatment combination in IGF1R studies[276].
g: genomic DNA; K: control; O: ODN2088 CpG oligonucleotide; P: picropodophyllin; C: chloroquine

4.3 NanoString and Taqman gene expression analyses (HGFR studies)

In relation to the expression of TLR9 mRNA, the application of g-, f- and mDNA treatments led to *TLR9* upregulation in comparison to the untreated control cells (**Figure 20/A**). *HGFR* gene expression was not upregulated in response to gDNA treatment when compared to the untreated control group. However, fDNA and mDNA treatments upregulated *HGFR* gene expression. When not incubated with gDNA, the gene expression profile remained comparable to that observed in the control samples (**Figure 20/A**). With the exception of IL1-β, all observed transcripts exhibited increased mRNA expression in response to fDNA administration. Genes associated with extrinsic and intrinsic apoptosis, including *Bcl-2*, *CD95,* and caspase-3, exhibited significant upregulation. Genes related to autophagy (*ULK1*), TLR9 signaling (*TRAF6*), c-Met signaling/anti-apoptotic factors (*PI3K* and *HGFR*), and apoptosis (*CD95L*) showed moderate upregulation. Conversely, genes related to autophagy (*ATG16L1, MAP1LC3B, Beclin-1*), pro-apoptotic mechanisms (*AMPK*), *HGFR* signaling, and *STAT3* displayed only modest upregulation (**Figure 20/A**). When incubated with mDNA, the anti-apoptotic *Bcl-2* gene exhibited significant upregulation. Conversely, the autophagy-related (*MAP1LC3B*), *TLR9*-signaling (IL8, *MyD88*), pro-apoptotic (*MAPK*), anti-apoptotic (*Akt*), and *c-Met*-signaling (*HGFR*) genes displayed moderate overexpression (**Figure 20/A**).

In relation to the impact of modified DNA treatments and combined HGFR inhibition on canonical and non-canonical *HGFR* signaling, it was observed that the concurrent administration of DISU and gDNA led to upregulation of *STAT3* and *CD95*, a marginal upregulation of *PI3K,* and a downregulation of *HGFR* expression (**Figure 20/B**). The simultaneous administration of fDNA and DISU increased the expression of *HGFR*, decreased the expression of *STAT3* and *PI3K*, and did not change the expression of *CD95* (**Figure 20/C**). The co-administration of DISU and mDNA resulted in increased expressions of *STAT3* and *HGFR* as well as decreased expressions of *PI3K* and *CD95* (**Figure 20/D**).

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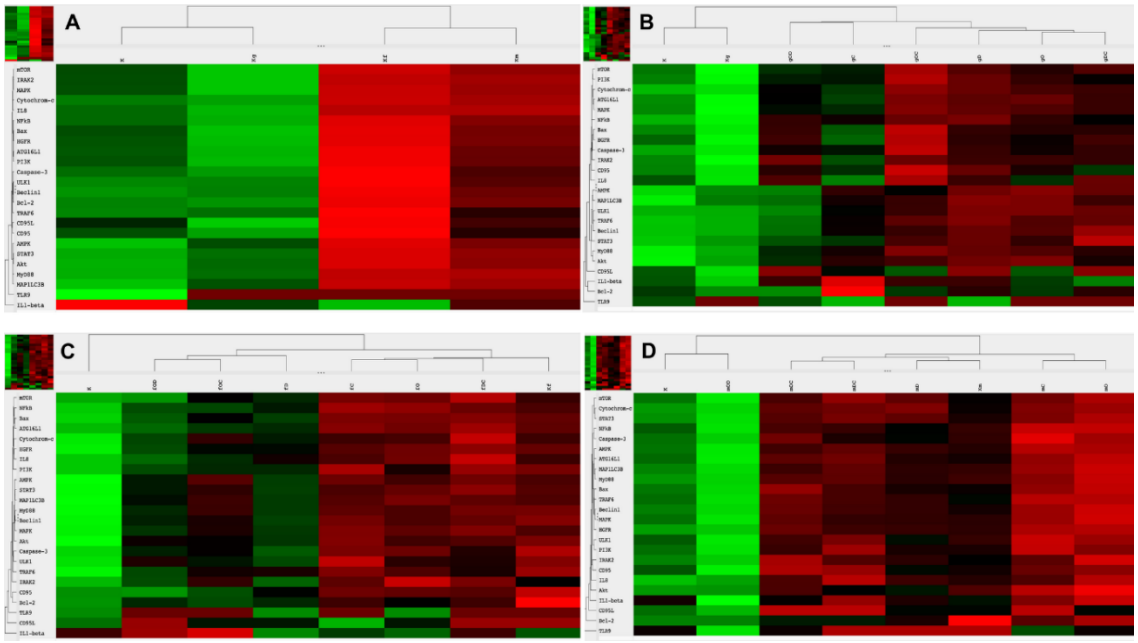

FIGURE 20 | Heatmap visualization of the NanoString gene expression analyses in HGFR studies [248].
A. Gene expression changes of modified DNA treatments as compared to control, non-treated HT29 cells. Gene expression alterations in HT29 cell after incubation with gDNA (**B.**), fDNA (**C.**) and mDNA (**D.**). g/f/m: genomic/fragmented/hypermethylated DNA; K: control; O: ODN2088 CpG oligonucleotide; D: DISU; C: chloroquine; red: overexpression, green: downregulation

Due to the fact that cfDNA treatment influences both *TLR9*-signaling and the autophagy apparatus, we also examined how inhibiton of *TLR9*-signaling or autophagy modifies the effect of concurrent HGFR inhibition and modified DNA treatment. Co-administration of all types of modified DNAs and DISU decreased the expression of genes implicated in canonical and non-canonical *HGFR* signaling by inhibiting *TLR9* signaling (**Figure 21/A**). The co-administration of gDNA and DISU, along with the inhibition of autophagy, did not affect the overexpression of *STAT3*, but it did reduce the expression of all other genes implicated in *c-Met* signaling (**Figure 21/A**). The simultaneous introduction of DISU and chloroquine along with fDNA or mDNA significantly increased the expression of each component of *HGFR* signaling (**Figure 21/A**).

In regard to genes associated with autophagy, the concurrent administration of *HGFR* inhibition and modified DNA treatments led to the upregulation of *ATG16L1*, *MAPLC3B*, *Beclin-1* and *ULK1*, with the exception of fDNA and *Beclin-1,* and mDNA and *ULK1*, for which there was no significant alteration in gene expression when compared to the control group (**Figure 21/B**). The concurrent inhibition of *HGFR*, modified DNA, and TLR9 led

to the downregulation of all genes associated with autophagy when gDNA or mDNA were utilized for treatment. Conversely, *MAPLC3B*, *Beclin-1,* and *ULK1* showed upregulation and incubation with fDNA did not result in any alteration in the expression of *ATG16L1*. Combining DISU, chloroquine, and modified DNAs led to the upregulation of all autophagy-associated genes (**Figure 21/C**).

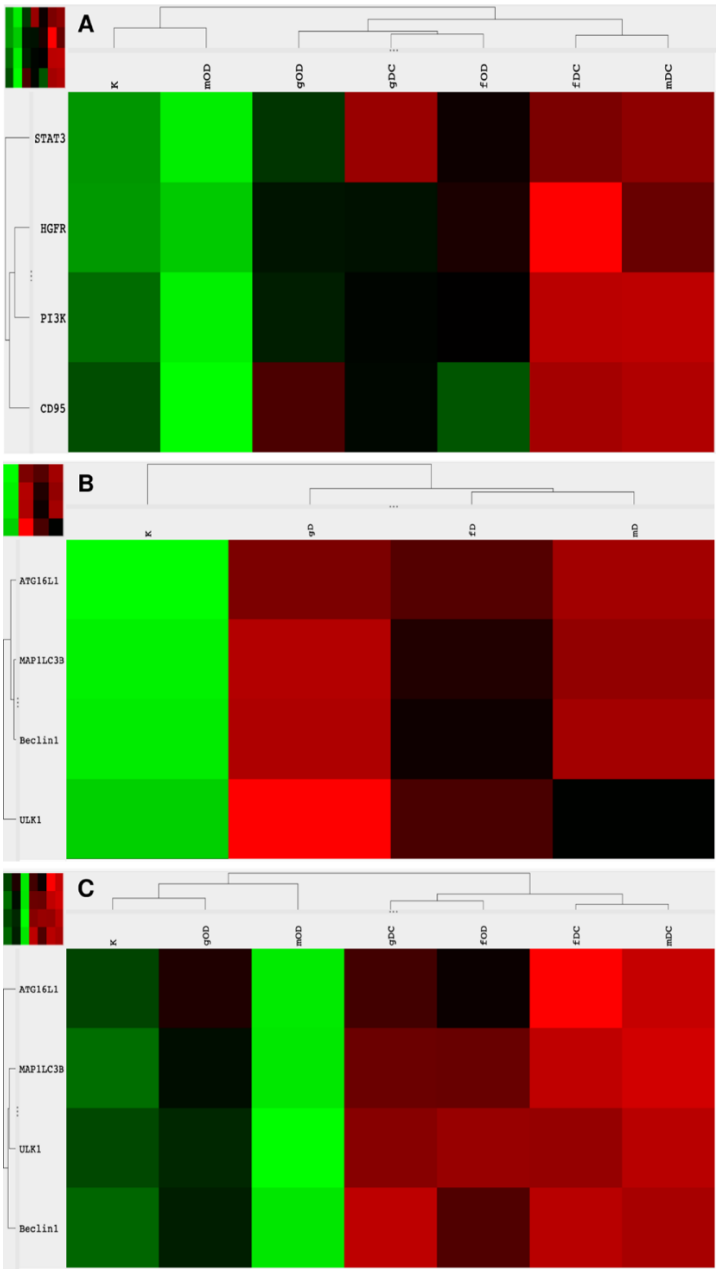

FIGURE 21 | Heatmap visualization of the NanoString gene expression analyses in HGFR studies: alterations in c-Met signaling pathways and autophagy [248].
Gene expression changes of combined treatments with modified DNAs, ODN2088, DISU, or chloroquine. g/f/m: genomic/fragmented/hypermethylated DNA; K: control; O: ODN2088 CpG oligonucleotide; D: DISU; C: chloroquine; red: overexpression, green: downregulation

The Taqman RT-PCR results confirmed the identification of changes in gene expression by NanoString/nCounter analysis. **Figure 22** provides a summary of the fold changes and **Supplementary Table 2** of SD values of the gene expressions that were analyzed.

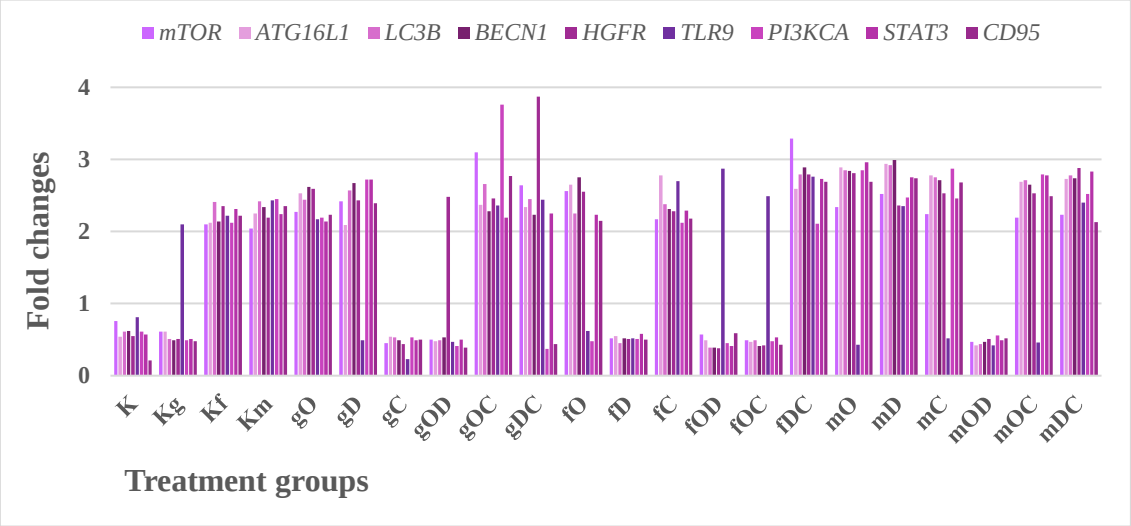

FIGURE 22 | Graphical visualization of the Taqman fold changes in HGFR studies [248].
Conventional Taqman RT-PCR confirmed the Nanostring results, with examined genes observed in all treatment groups ($p<0.05$; n=3).
g/f/m: genomic/fragmented/hypermethylated DNA; K: control; O: ODN2088 CpG oligonucleotide; D: DISU; C: chloroquine

4.4 NanoString and Taqman gene expression analyses (IGF1R studies)

In terms of TLR9 mRNA expression, gDNA treatment led to an upregulation of TLR9 in comparison to the untreated control cells. In comparison to the untreated control, gDNA treatment had no effect on the expression of the *IGF1R* gene. When not incubated with gDNA, the gene expression profile was comparable to that observed in the control samples.

With respect to the impact of gDNA treatment and combined IGF1R inhibition on the expression of *IGF1R* signaling elements, a marginal increase in *IGF1R* expression was observed; however, there was no significant alteration in the expressions of *MAPK*, *PI3K,*

and *Akt*. The combination of gDNA with ODN2088 or chloroquine significantly overexpressed all examined genes, except for *TLR9* in gC and *Bcl-2* in gO.

Due to the fact that cell-free DNA treatment influences both *TLR9* signaling and the autophagy apparatus, we also investigated how inhibiting *TLR9* signaling or autophagy modifies the effect of concurrent IGF1R inhibition and gDNA treatment.

TLR9 signaling-related (e.g., *MyD88*, *NF-κB*), autophagy-related (e.g., *ATG16L1*, *Beclin-1*, *MAP1LC3B*, *ULK1*, *Ambra-1*), autophagy suppressor/anti-apoptotic (e.g., *PI3K*, *Akt*, *mTOR*) and autophagy activator/pro-apoptotic (e.g., *MAPK*, *AMPK*, *Bax*) gene expressions increased most significantly when gDNA was combined with picropodophyllin and chloroquine, with the exception of *TLR9* and *Bcl-2*. Conversely, the utilization of gDNA in conjunction with ODN2088 and picropodophyllin led to a widespread suppression of the examined genes, except for *TLR9* and *Bcl-2*. All four treatment combinations (gP, gC, gO, and gPC) increased the activity of the gene CD133, which is associated with cancer stemness. **Figure 23** depicts the visible modifications in gene expression. The Taqman RT-PCR results confirmed the identification of changes in gene expression by NanoString/nCounter analysis. **Figure 24** and **Supplementary Table 3** present a summary of the fold changes of the analyzed gene expressions.

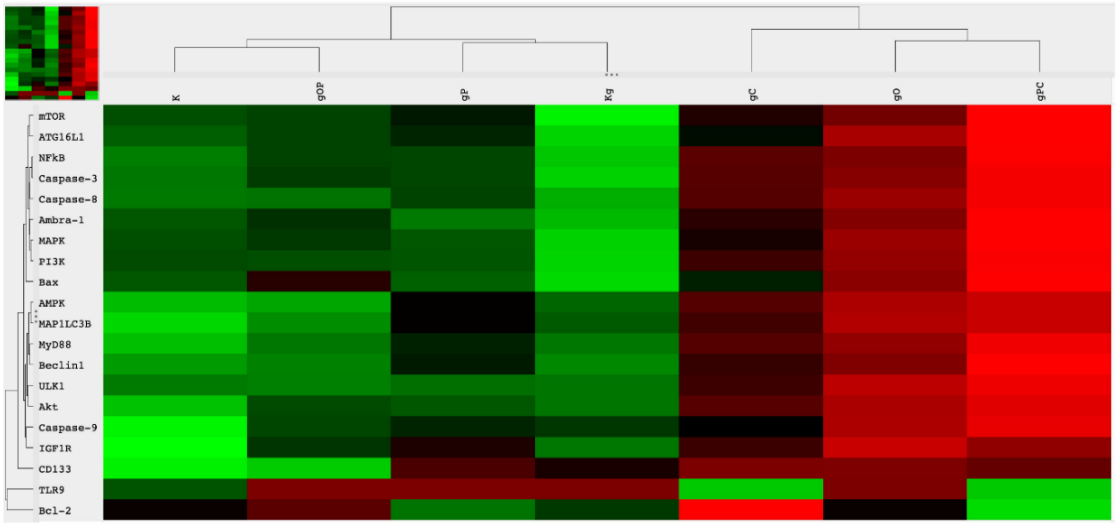

FIGURE 23 | Heatmap visualization of the NanoString gene expression analysis in IGF1R studies [276].
Gene expression alterations in HT29 cells after incubation with genomic self-DNA.
g: genomic DNA; K: control; O: ODN2088 CpG oligonucleotide; P: picropodophyllin; C: chloroquine; red: overexpression, green: downregulation

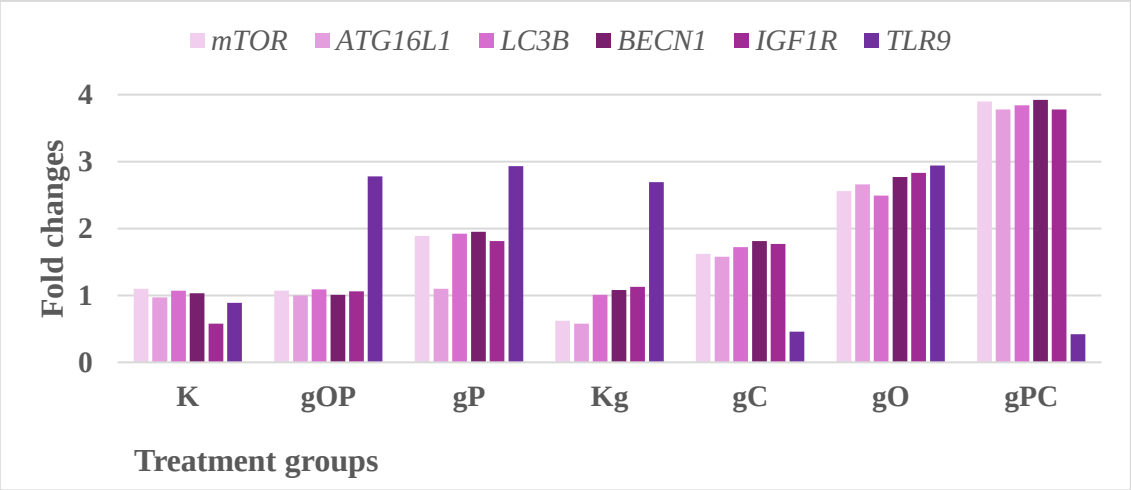

FIGURE 24 | Graphical visualization of the Taqman fold changes in IGF1R studies [276].
The gene expression fold changes were in correlation with the NanoString gene expression results ($p<0.05$; n=3).
g: genomic DNA; K: control; O: ODN2088 CpG oligonucleotide; P: picropodophyllin; C: chloroquine

4.5 Immunocytochemistry and WES Simple (HGFR studies)

We performed immunocytochemistry in specific instances to validate gene expression findings at the protein level.

Untreated control HT29 cells showed mild to moderate TLR9 immunopositivity. TLR9 protein expression ranging from moderate to strong was observed subsequent to incubation with g-, f- and mDNAs. HGFR immunocytochemistry revealed a mild immunoreaction in control and gDNA-treated samples, while fDNA and mDNA treatments produced a strong immunopositivity. In relation to autophagy, the expression of ATG16L1, Beclin-1, and LC3 proteins was significantly increased in response to f- and mDNAs; moderate to strong immunoreactions were observed in these cases, in contrast to the untreated control group and HT29 cells treated with gDNA (**Figure 25**). The outcomes of the immunochemistry assay reflected those of the NanoString and Taqman gene expression assays.

Variations in LC3B protein levels among the groups under investigation were consistent with changes in gene expression as measured by NanoString and Taqman as well as immunocytochemistry. In relation to autophagy, the protein levels of LC3B and p62 indicate that the combined application of DNA treatments (g, f and m) and DISU amplifies the inhibitory effect of chloroquine, specifically by inducing a greater inhibition

of autophagy. Conversely, the suppression of autophagy results in protein accumulation via the inhibition of LC3B and p62 degradation. **Figure 26** illustrates the outcomes of the WES Simple.

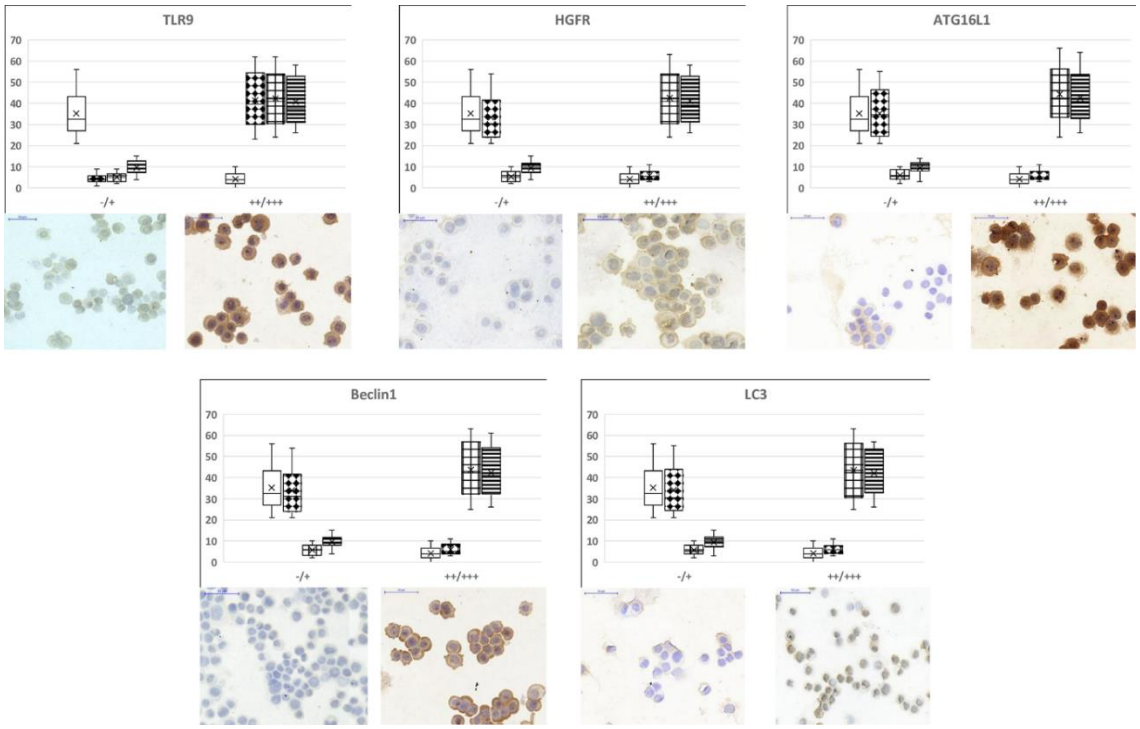

FIGURE 25 | TLR9, HGFR and autophagy-related protein immunocytochemistry results in HGFR studies [248].
The box and whisker plots represent the one-way ANOVA results of immunocytochemistry analyses. The percentage of non-immunoreactive and weakly immunopositive ("-/+"), as well as moderately and strongly immunopositive ("++/+++") HT29 cells within the treatment groups was visualized. Under the plots representative "-/+" and "++/+++" image inserts can be seen ($p<0.05$; n=3). Scale bars represents 50 µm. Empty boxes: control, non-treated cells; diamond dots boxes: gDNA treatment; square grid boxes: fDNA treatment; striped boxes: mDNA treatment

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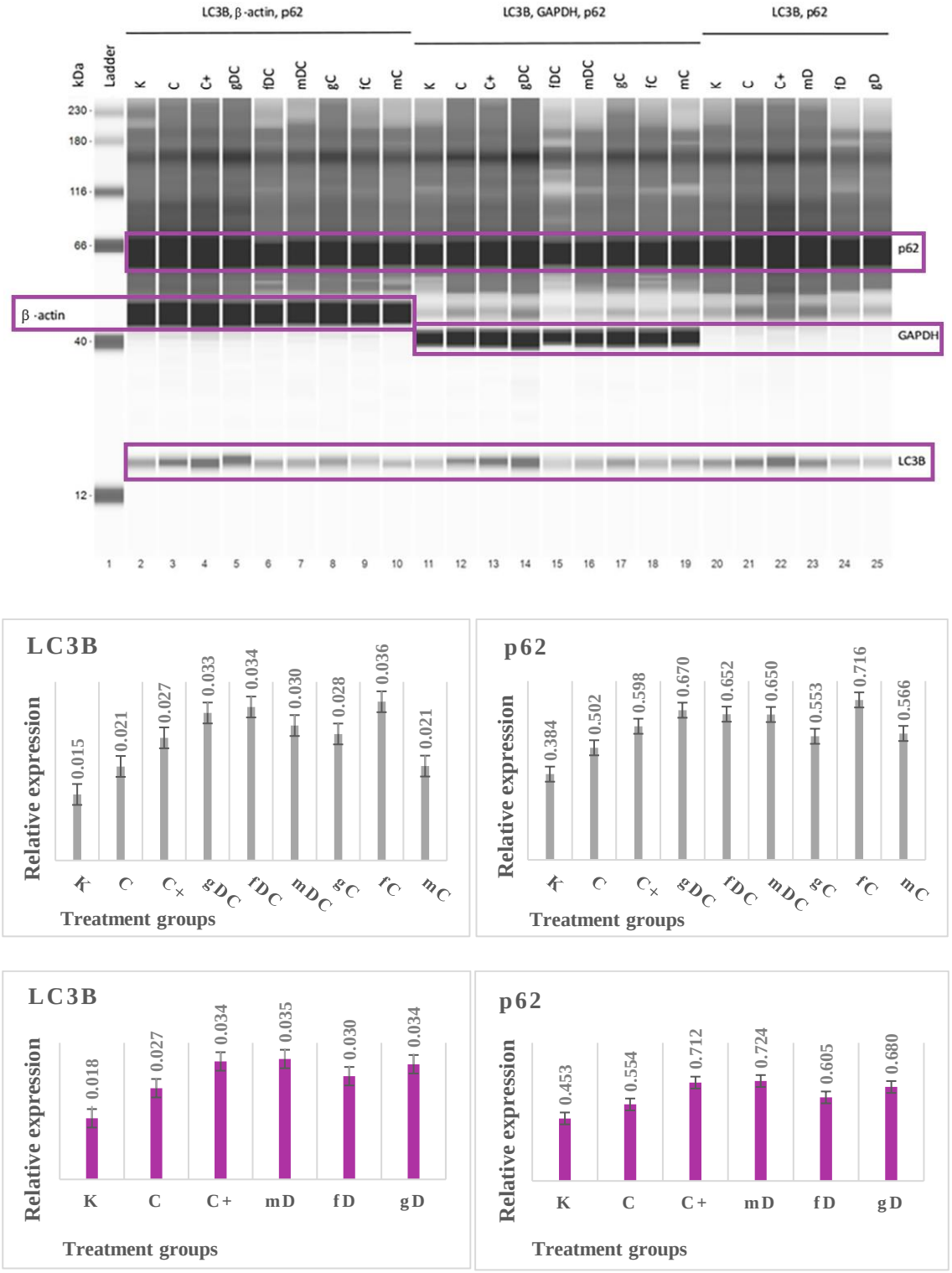

FIGURE 26 | Results of the p62/sequestrome 1 and LC3B WES Simple in HGFR studies [248].
The figure is a representative blot image. The graphs show the expression of proteins: the area of the tested proteins was multiplied by the values of the β-actin area ($p<0.05$; n=3).
g/f/m: genomic/fragmented/hypermethylated DNA; K: control; C: chloroquine (10 μM); C+: chloroquine (50 μM) D: DISU

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4.6 Immunocytochemistry and WES Simple (IGF1R studies)

We carried out immunocytochemistry on autophagy-related genes (*ACTN16L1*, *Beclin-1,* and *MAP1LC3B*) associated with IGF1R in order to validate protein-level gene expression results. The correlation between the gene expression results and the distribution of non-immunoreactive and weakly immunopositive ("-/+") HT29 cells, as well as moderately and strongly immunoreactive ("++/+++") cells, was the initial observation. An increased degree of moderate to strong immunopositivity was observed in the case of IGF1R subsequent to incubation with gO, gC, and gPC. Concerning autophagy, the gO and pPC groups exhibited the most pronounced upregulation of ATG16L1 protein expression, which was followed by the Kg, gP, and gOP interventions. The gO and gPC treatment groups showed the highest proportion of strong Beclin-1 and LC3 immunoreactivity, followed by the gP and gC treatments. We conducted an analysis of the NanoString gene expression results to determine whether or not each treatment group contained an HT29 cell expressing CD133 protein. CD133-positive cells were observed in the gO, gP, gC, and gPC treatment groups only in a dispersed manner. **Figure 27** illustrates the representative immunocytochemistry images and the outcomes of the one-way ANOVA test. **Supplementary Figure 1** illustrates the results of the Tukey HSD test.

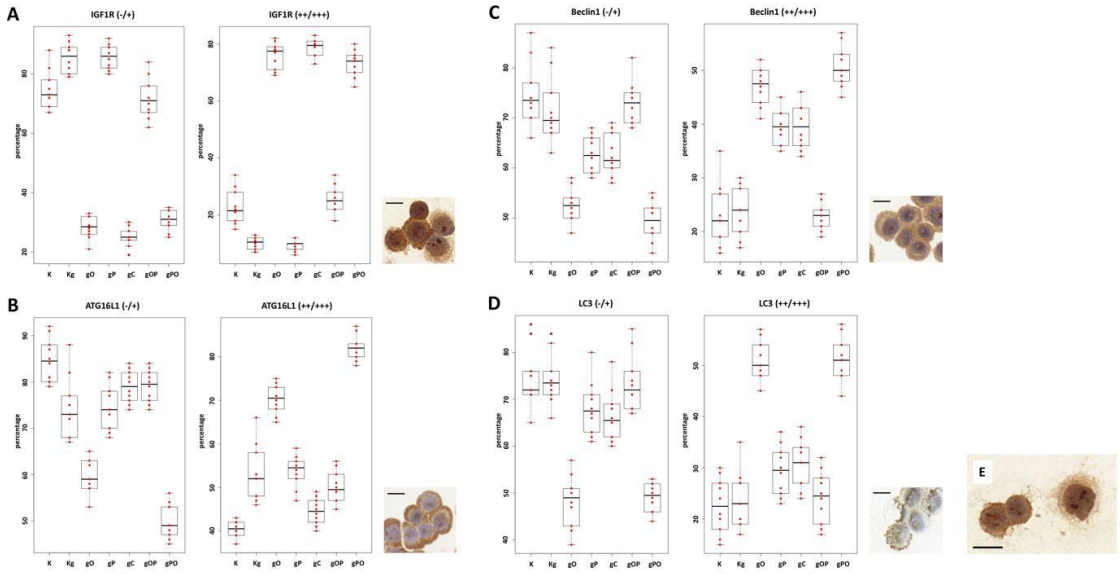

FIGURE 27 | One-way ANOVA results of IGF1R, ATG16L1, Beclin1, and LC3 immunocytochemistry analyses in IGF1R studies [276].
The percentages of non-immunoreactive and weakly immunopositive ("−/+"), as well as moderately and strongly immunopositive ("++/+++") HT29 cells within the treatment groups were plotted on box and whisker plots ($p<0.05$; n=3). (**A**) The boxplots for IGF1R. The right upper inserts represent the moderate to strong IGF1R immunopositivity (at ×200 magnification; the scale bar indicates 10 μm). The boxplots and representative immunostainings for ATG16L1 (**B**), Beclin1 (**C**) and LC3B (**D**) are also visualized. (**E**) Right lower insert represents CD133 positive HT29 cells (×200 magnification; the scale bar indicates 10 μm).
g: genomic DNA; K: control; O: ODN2088 CpG oligonucleotide; P: picropodophyllin; C: chloroquine

The inclusion of picropodophyllin in our experimental setup on gDNA-treated cells led to a comparatively modest reduction in the expression of *PI3K*, *Akt*, *AMPK,* and *mTOR* genes. This reduction may have had an inhibitory effect on autophagy initiation. *mTOR* has to be active if autophagy is inhibited; therefore, a WES Simple was also conducted. In the K, Kg, and gP groups, the gene expression results were linked to the levels of mTOR, phospho-mTOR, and autophagy-related proteins, as well as the activity of phospho-mTOR (**Figure 28**).

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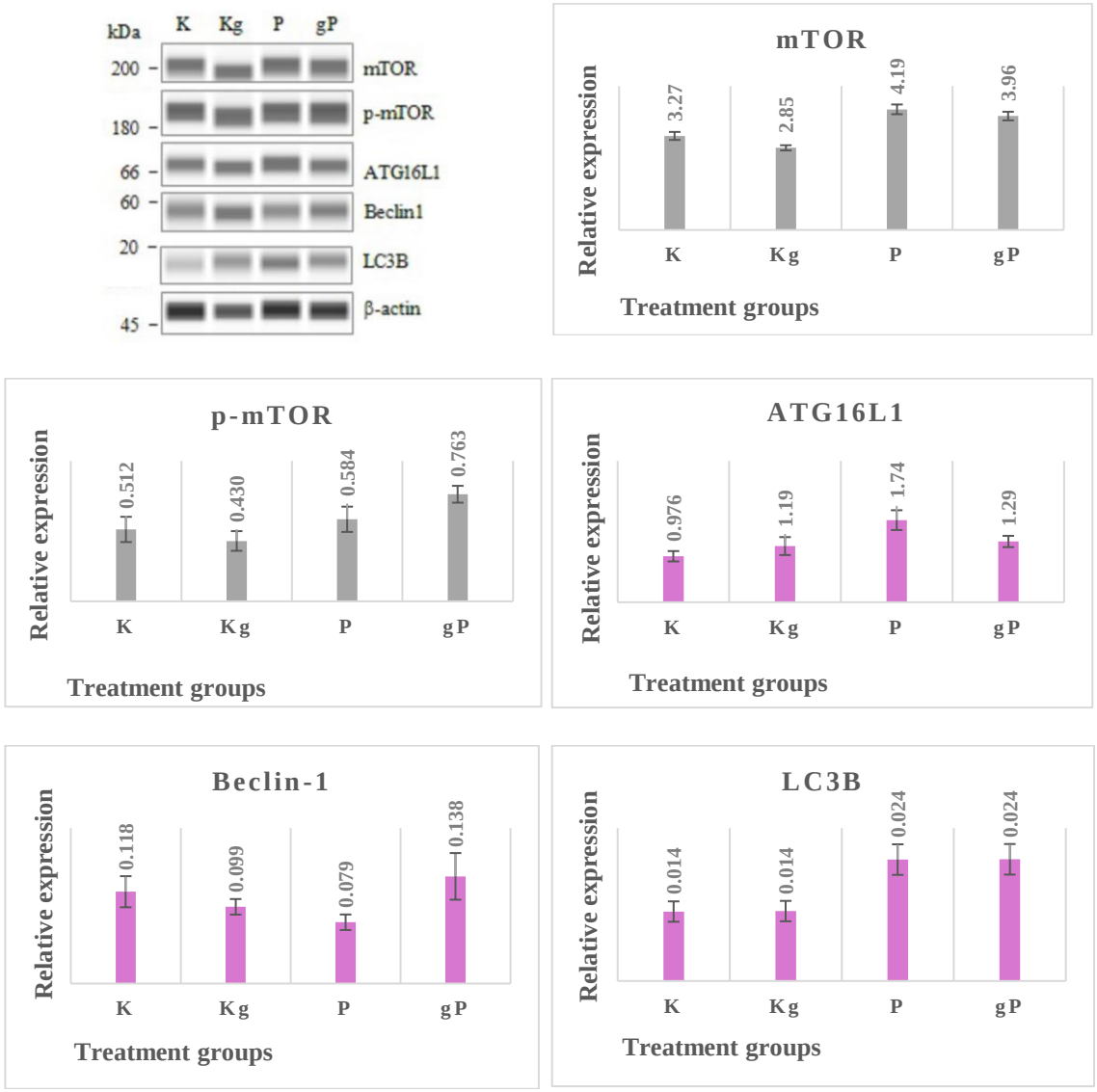

FIGURE 28 | WES Simple analyses of selected proteins in IGF1R studies [276].
According to protein expression values normalized to β-actin (bar graphs), the mTOR and phospho-mTOR (p-mTOR) protein activities (grey), as well as the autophagy-related protein (ATG16L1, Beclin-1, LC3B) expressions (purple) were in relation to the gene expression results ($p<0.05$; n=3).
g: genomic DNA; K: control; P: picropodophyllin

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4.7 Transmission electron microscopy (HGFR studies)

In the cytoplasm of control, untreated, metabolically active HT29 cells, autophagic vacuoles (AV) were seen (3±1 pcs/cell), which showed the same phenotype as chloroquine-treated controls (4±1.5 pcs/cell), indicating the occurrence of macroautophagy. The incidence of AVs was higher in DISU (6±1.8 pcs/cell) and ODN2088 (7±1.4 pcs/cell) compared to control cells. A more pronounced form of macroautophagy was observed upon incubation with gDNA (6±2 pcs/cell). Additionally, the simultaneous administration of chloroquine (7±1.6 pcs/cell), ODN2088 (9±1.2 pcs/cell), and DISU (7±2 pcs/cell) further promoted the presence of intensive autophagy. After the administration of fDNA (5±1.8 pcs/cell), multivesicular bodies (MVBs) and AVs both appeared. The autophagy process was significantly enhanced by the combined effect of ODN2088 and fDNA (12±2 pcs/cell) (fD: 6±1.4 pcs/cell; fC: 4±2.3 pcs/cell). However, the cells disintegrated when administered with fDNA and any inhibitor simultaneously. After incubation with mDNA (7±1.3 pcs/cell), the cells showed chromatin condensation and blebbing, in addition to disorganization. The introduction of chloroquine into mDNA (5±1.6 pcs/cell) led to the formation of MVBs. However, the combination of mD (7±1.4 pcs/cell) improved cell survival, and the activated macroautophagy appeared to aid in the maintenance of cellular fitness. The combination of mO generated the fewest number of AVs (2±1.3 pcs/cell). Following this, autophagy was detected to a different extent in each cohort of HT29 cells. **Figure 29** illustrates the representative microstructural alterations.

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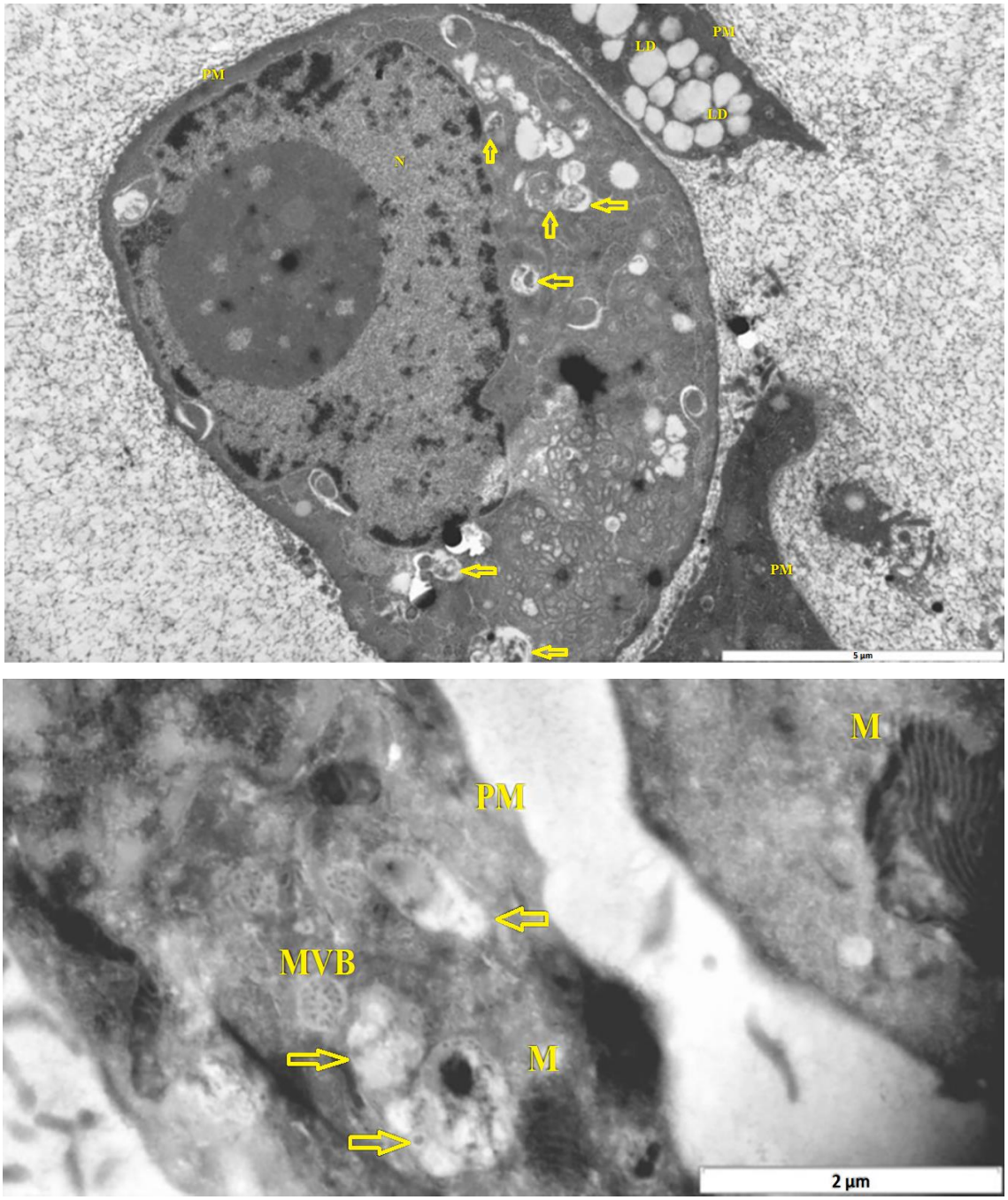

FIGURE 29 | Transmission electron microscopy results of HGFR inhibition [248].
The representative images highlight the autophagy-related structural changes in HT29 cells (from top to down: disorganized nucleus with chromatin condensation plus autophagic vacuoles (scale bar: 5µm); autophagic vacuoles with multivesicular body (scale bar: 2 µm)). ($p<0.05$; n=3).
Arrows: autophagic vacuoles; MVB: multivesicular body; PM: plasma membrane; N: nucleus; M: mitochondria; LD: lipid droplet

4.8 Transmission electron microscopy (IGF1R studies)

Control, non-treated, metabolically active HT29 cells (3±1 pcs/cell), similarly to chloroquine-treated controls (4±1.5 pcs/cell), displayed autophagic vacuoles (AVs) in the cytoplasm, indicating macroautophagy. The frequency of AVs in ODN2088 (7±1.4 pcs/cell) control cells was higher as compared to control. However, in picropodophyllin-treated control cells, AVs were only scattered (0.5±0.5 pcs/cell). Incubation with gDNA resulted in the appearance of a more intense macroautophagy (6±2 pcs/cell), and co-administration of ODN2088 (10±2.2 pcs/cell) or chloroquine (5±1.5 pcs/cell) also favored the presence of intense autophagy, represented by disorganized cell structure along with chromatin condensation and blebbing. The combination of gDNA with picropodophyllin and/or ODN2088, similarly to non-treated control cells, resulted in a low number of AVs (2±1.5 pcs/cell in gP; 3±1 pcs/cell in gOP). On the contrary, gPC co-treatment caused an intense macroautophagy (11±2.6 pcs/cell). The gP combination resulted in the detection of multivesicular bodies (MVBs). Thus, the presence of autophagy was observed in each group of HT29 cells but to different extents. The representative microstructural changes together with the numerical data can be seen in **Figure 30**.

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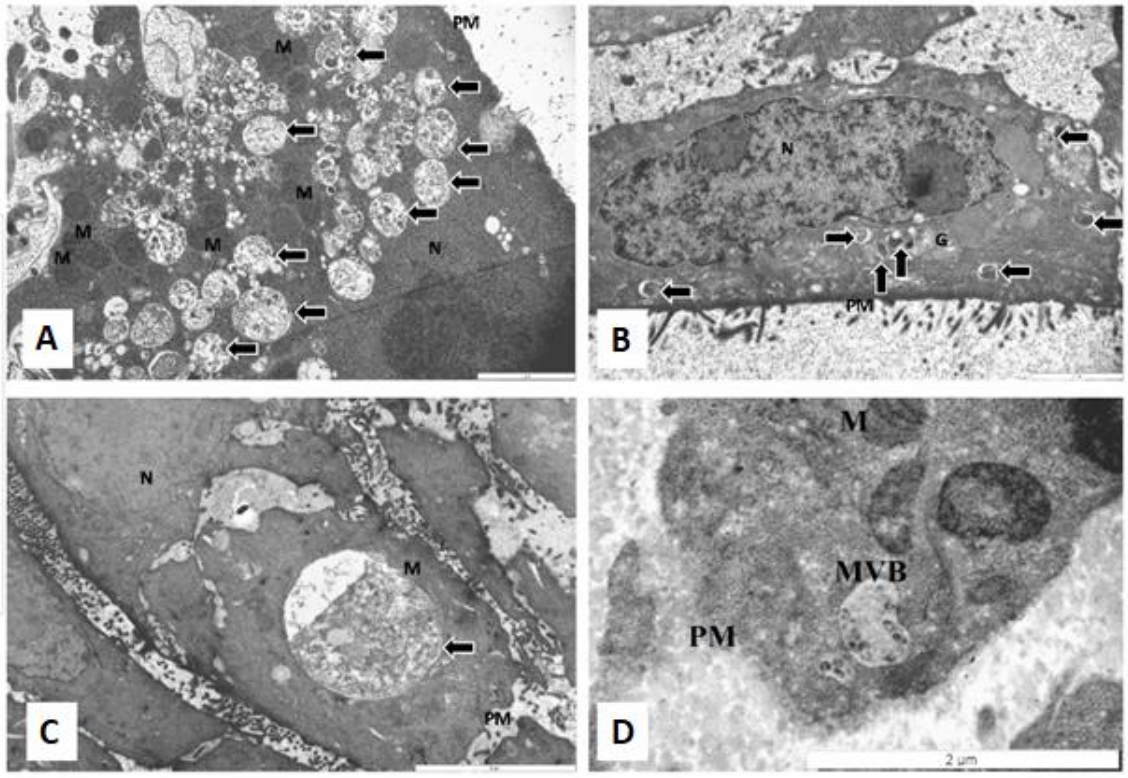

FIGURE 30 | Transmission electron microscopy results of IGF1R inhibition [276].
The representative image inserts highlight the autophagy-related structural changes in HT29 cells; (**A**) large number of AVs in gPC (scale bar: 2 µm); (**B**) large late-stage AV in gO (scale bar: 5 µm); (**C**) disorganized nucleus with chromatin condensation (scale bar: 2 µm); (**D**) multivesicular body in gP (scale bar: 2 µm). ($p<0.05$; n=3).
Arrows: autophagic vacuoles; MVB: multivesicular body; PM: plasma membrane; N: nucleus; M: mitochondria

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4.9 Semithin sections (HGFR studies)

In certain cases, semithin sections were also examined to determine whether the decrease in cell numbers following treatment with modified self-DNAs and/or inhibitors of TLR9, HGFR, or autophagy was due to increased cell mortality or decreased proliferation activity. When incubated with mDNAs, gDNAs, or fDNAs, the number of proliferating cells was directly proportional to the incidence of proliferation. Co-administration of gDNA with DISU and chloroquine resulted in exceptionally reduced proliferative activity. The combination of mDNA with ODN2088 and DISU led to increased proliferative activity, as shown in **Figure 31 A-F**.

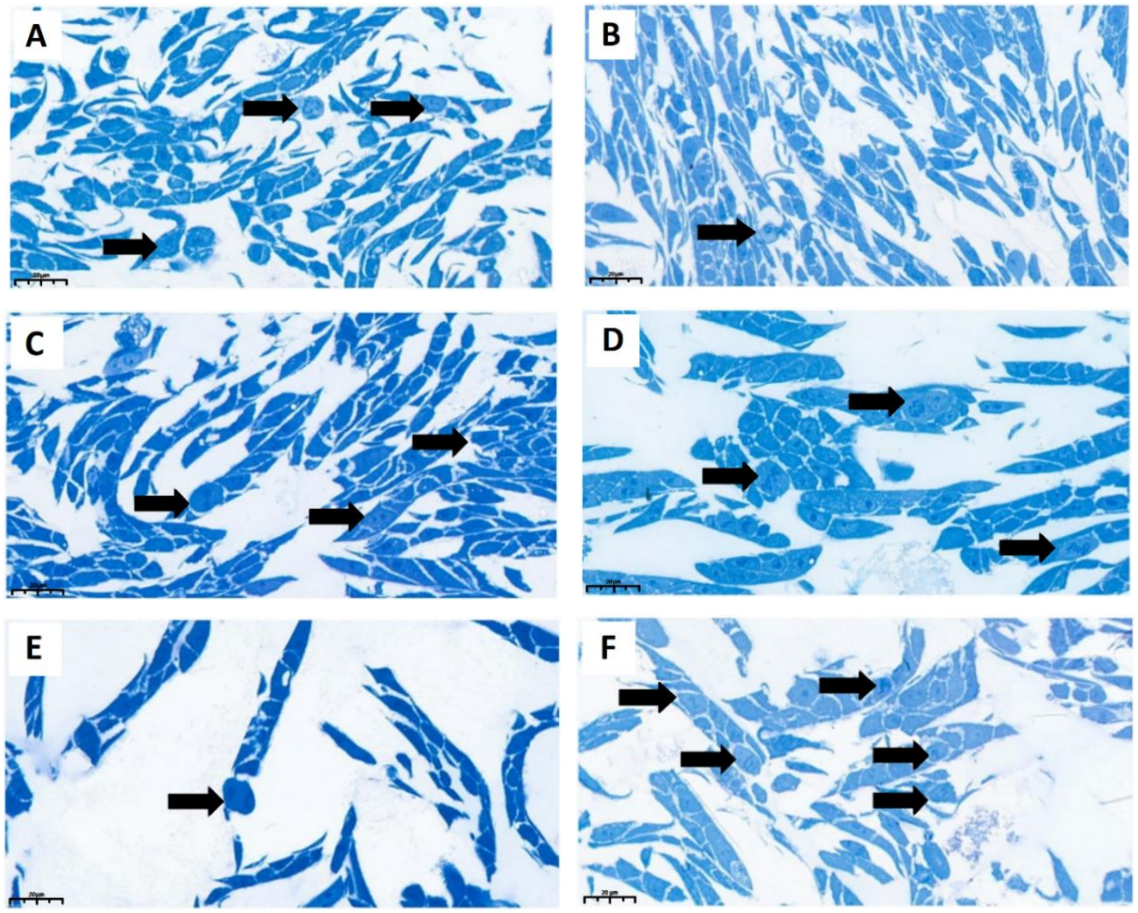

FIGURE 31 | Signs of proliferative activity in HT29 cells in HGFR studies [248].
In case of gDNA, DISU and chloroquine co-treated, the number of cell divisions was decreased. After mDNA, ODN2088 and DISU treatment, the proliferation activity of HT29 cancer cells increased. Arrows indicate cell divisions; scale bar represents 20 μm; $p<0.05$; n=3.
K (**A**); gDNA (**B**); fDNA (**C**); mDNA (**D**); gDC (**E**); mOD (**F**)

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4.10 Semithin sections (IGF1R studies)

In order to ascertain whether the alterations in cell counts subsequent to interventions involving genomic DNA and/or inhibitors of *TLR9*, *IGF1R*, or autophagy were attributable to decreased proliferation activity or increased cell mortality, semi-thin sections of specific cases were also analyzed. Using gDNA for incubation led to a direct correlation between the number of cells obtained and the incidence of proliferation. Co-administration of gDNA with picropodophyllin and/or chloroquine resulted in exceptionally diminished proliferative activity. **Figure 32 A-C** illustrates that increased proliferative activity was observed when gDNA was combined with ODN2088 and picropodophyllin Combining gDNA with ODN2088 and picropodophyllin led to increased proliferative activity, as shown in **Figure 32 A-C**.

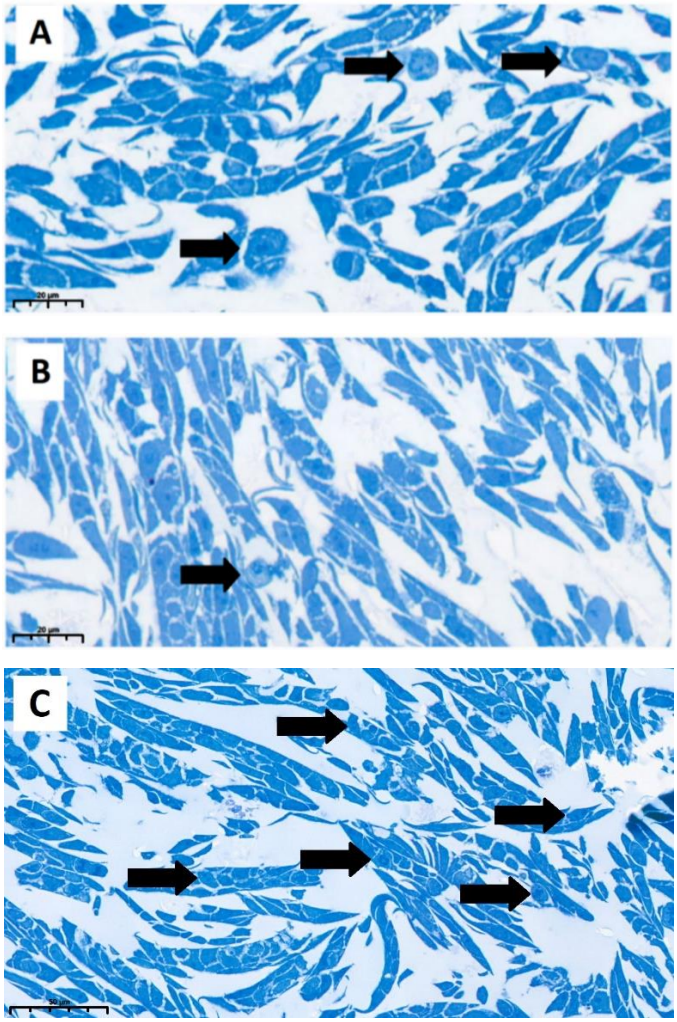

FIGURE 32 | Cell divisions in HT29 cells in IGF1R studies [276].
A Control, non-treated cells; (**B**) gDNA-treated control cells; (**C**): larger number of cell divisions in gOP sample. Arrows indicate cell divisions; scale bar represents 20 μm; $p<0.05$; n=3.

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5. DISCUSSION

5.1 The interconnection of TLR9-mediated autophagy response and HGFR signaling

Our aim was to determine the impact on HT29 cell viability and proliferation of inhibiting TLR9 signaling, autophagy, and/or HGFR signaling in conjunction with intact or modified tumor self-DNA treatment.

Initially, we evaluated the impact of self-DNA-induced TLR9 signaling modulation on the survival of HT29 cells. It is established knowledge that cfDNA sequences can be detected in human blood, urine, saliva, and feces [1]. The methylation status or fragmentation of cfDNAs may encode information about their origin [287,288]. Concerning their source, cfDNAs can be classified into various groups. Endogenous cfDNA sequences originate from cellular and tissue components, whereas exogenous sequences are predominantly obtained from sources such as the host microbiome, infectious agents, the embryo, and diet [288-291].

TLRs are the innate immune system's receptors. DNA can be detected by TLR9 from both exogenous and endogenous sources [292]. Self-DNA structural modifications (i.e., fragment length and methylation status) have been shown to significantly influence the activation of TLR9-mediated signaling pathways [119,293].

The constitutive expression of TLR9 mRNA in HT29 cells was previously documented [294]. The expression of TLR9 mRNA is minimal at baseline, but it is significantly upregulated through incubation with CpG-ODN or tumorous self-DNA [119,294]. Additionally, we also observed that the expression of the TLR9 gene was augmented in cell populations that were exposed to genomic, fragmented, or hypermethylated self-DNAs, in comparison to the control group of untreated HT29 cells.

Autophagy can be induced in tumor cell lines (e.g., colon, breast, and prostate malignancies) in a manner dependent on the expression of TLR9 induced by CpG-ODNs [167]. Gene expression changes in glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the production of reactive oxygen species (ROS) may act as signals between TLRs and autophagy [295-297]. *TLR9* and autophagy have several features in common, including their respective impacts on cellular survival and demise, their involvement in innate immunity, the facilitation of MHC class II antigen presentation,

their interactions within endosomes, the beneficial influence of class III *PI3K* on their signaling, and the inhibitory effects of common substances (hydroxychloroquine, 3-methyladenine, and bafilomycin A1) [167]. A close relationship between TLR9 signaling and the autophagy response has been demonstrated to have remarkable effects on the survival of HT29 cells treated with modified self-DNA [293].

In our research, treatments with modified self-DNA altered HT29 cells' metabolic activity and proliferation to varying degrees. It is noteworthy that the noted decrease in the expression levels of all genes tested was attributed to gDNA, while an increase in the expression levels of fragmented and hypermethylated DNAs was observed. This may be the result of the investigated components of the *TLR9* signaling pathway demonstrating both pro- and anti-survival effects [293,298-302] and the activation of this complex signaling pathway by self-DNA sequences with distinct modifications [293].

In all cases where cells were treated with modified self-DNAs, inhibiting *TLR9* signaling led to increased metabolic activity. Incubation with genomic and hypermethylated DNAs resulted in a reduction in cell division, whereas treatment with fragmented DNA marginally enhanced cell proliferation. The reduction of *TLR9* signaling nevertheless nullified the impact of self-DNA treatments. Aside from the role of distinct levels of TLR9 signaling activation, the differential expression of pro- and anti-apoptotic genes can be hypothesized to be in the background of this observation [303-307].

The subsequent procedures involved examining the impact of changes in the interaction between the *HGFR* and *TLR9* signaling pathways on the survival of HT29 cells. There is a lack of information available about this intricate signaling crosstalk. The activation of TLR2 and TLR5 in epithelial cells has been shown to recently induce phosphorylation of RTKs that are involved in the growth, repair, and carcinogenesis of the epithelium. In addition to all epidermal growth factor receptor (EGFR) family members, TLR stimulation can also activate other RTKs, including HGFR [167]. Chemotactic stimuli and *TLR-MyD88* signaling have the potential to activate extracellular signal-regulated kinases (ERKs). Growth hormones can stimulate ERKs, which in turn can stimulate RTKs. Specifically, both *TLR-MyD88*-dependent and *TLR-MyD88*-independent ERK activation in macrophages infected with *Toxoplasma gondii* has been described [308]. Given that *MyD88* is a crucial component of the *TLR9* signaling pathway,

the possibility of a molecular connection between the *TLR9* and *HGFR* signaling pathways cannot be ruled out.

Our findings indicate that self-DNA treatment has a tendency to increase *MyD88* expression and that HGFR inhibition does not alter or further enhance this effect. We found a correlation between *MyD88* overexpression and increased HT29 cell proliferation. Recent research indicates that *MyD88* inhibits apoptosis in colon carcinoma cells via the *Ras/Erk* pathway but not via the *NF-κB* pathway [309]. A comparable alteration in the expression of caspase-3 was noted to that of *MyD88*. Inhibited apoptosis-induced compensatory cell proliferation, which is significantly influenced by caspases (e.g., caspase-3), may therefore account for a portion of the observed changes in HT29 cell proliferation [310].

Additionally, we investigated the impact of the interaction between *TLR9* and *HGFR* signaling on HT29 cell proliferation and autophagy. Strict regulation of RTK trafficking is essential for maintaining homeostasis. RTKs prevent access to degradation pathways in human malignancies [311]. Besides growth factor RTKs, signaling cascades and nutrient availability fluctuations have been demonstrated to regulate autophagy [312]. It was discovered that autophagy mediated by *LC3C* regulates HGF/HGFR-stimulated migration and invasion in HeLa cancer cells in a selective manner [279]. In relation to the interaction between *HGFR* signaling and autophagy machinery in colorectal cancer cell lines, recent research has revealed that basal autophagy, independent of mTORC1 positively regulates the phosphorylation levels of multiple RTKs, including HGFR. Furthermore, research has shown that genetic inhibition of basal autophagy reduces *Akt* activation mediated by mTORC2 but has no discernible impact on mTORC1 activity. Additionally, it has been shown that autophagy regulates mTORC2 and positively mediates the phosphorylation of HGFR; the impaired HGFR phosphorylation in autophagy-deficient cells was due to decreased mTORC2 activation [280].

All types of modified self-DNA treatments increased autophagy gene expression, but it was fDNA administration that specifically increased cell growth. Autophagy was disrupted due to the accumulation of p62 and LC3B proteins caused by the combination of DISU and DNA. In contrast to DNA alone, the combined effect of DISU and DNA was antithetical to that of DNA in relation to cell proliferation. In recent times, scholars have uncovered a multifaceted reciprocal association between autophagy-master regulator

kinases and autophagy-associated proteins [313]. HGFR and β1-integrin colocalize with compartments that are positive for *Beclin-1* and/or *LC3B*. Additionally, in response to HGF stimulation, a concentration of phosphorylated ERK1/2 localizes alongside HGFR in endomembranes associated with autophagy [314]. HGFR has also been seen to partially colocalize with LC3B-positive perinuclear vesicles, which may have an effect on its phosphorylation. This is because HGFR phosphorylation is only enhanced under autophagy-competent conditions [280], which is precisely when chloroquine-mediated accumulation of autophagosomes occurs. As a result, autophagic vesicles may serve as signaling platforms through which mTORC2 regulates HGFR phosphorylation [313].

The most significant inhibition of cellular proliferation was observed when gDNA, DISU, and chloroquine were utilized concurrently. An instance of *STAT3* overexpression was identified, which is implicated in non-canonical *c-Met* signaling. *STAT3* activity in colon carcinoma cells is stimulated by IL-6 or promoted by cancer cell multiplication by a constitutively active *STAT3* mutant [315]. Based on these factors, *STAT3* stimulates cellular proliferation. IL-6 production is also stimulated by TLR9 activation [316], which is advantageous for *STAT3* activation. Conversely, *LC3B* exhibited upregulation within this cohort of HT29 cells. The threshold for *LC3B* activation rises in the event of *LC3B* upregulation, a factor that can regulate pro-apoptotic function [317]. It is not impossible that the effect of *LC3B* on stopping cell growth overpowered the effect of *STAT3* expression on encouraging cell growth in this case, resulting in a significant decrease in cell growth. Additionally, we observed the accumulation of *LC3B* and *p62* proteins in the gDC group. This implies that autophagy dysfunction was the consequence of the combined therapeutic interventions. Proliferation ability is enhanced when there is an excessive accumulation of *p62* in tumor cells, which is characterized by cell cycle initiation and inhibition of apoptosis [318]. On the contrary, a recent study [319] found that in CRC patients, high expression of LC3B dot-like and p62 dot-like cytoplasmic proteins (which indicate impaired autophagy) was associated with the best prognosis. These results indicate that inhibiting autophagy is associated with a reduction in cell proliferation. It is important to note, however, that while previous studies have documented chloroquine's ability to eliminate cancer cells without inhibiting autophagy [320-322], the 10 μM chloroquine we utilized in this study merely inhibited autophagy without impacting the proliferation of HT29 cells. On this basis, it is possible to

hypothesize that the concurrent administration of gDNA and DISU along with chloroquine contributed to the observed reduction in cell proliferation within the gDC group.

In gastric adenocarcinoma cells, it was discovered that the induced form of cytoprotective autophagy in response to HGFR inhibition or a combination of HGFR and autophagy inhibition can lead to a substantial reduction in cell viability [323]. It has been demonstrated that the administration of HGF and mTOR agonist MHY1485 can inhibit autophagy induction as well as the dephosphorylation of *ULK1* and *mTOR*, which occurs in response to HGFR inhibitor treatment. This suggests that the HGFR inhibitors induced autophagy via the *HGFR-mTOR-ULK1* molecular cascade. It is noteworthy that HGFR inhibitors exhibited additional suppression of cell survival and tumor growth in Met-amplified cancer cells when autophagy was inhibited. Therefore, HGFR inhibitor-mediated autophagy appears to be mediated by the *HGFR-mTOR-ULK1* cascade; therefore, HGFR inhibitors combined with autophagy inhibitors may represent a promising therapeutic approach for Met-amplified malignancies [240].

Concurrent administration of mDNA, ODN2088, and DISU detected a maximum degree of cellular proliferation. Remarkably, in this instance, both canonical and non-canonical *HGFR* signaling pathway expression was reduced, in addition to autophagy-related gene expression. It has been shown that decreased autophagy promotes cell proliferation through an unidentified mechanism [324-326]. Genetic silencing of critical autophagy proteins (e.g., *Beclin-1*, *Ambra 1*) in mice has been shown to result in increased cell proliferation [326]. It is not impossible to rule out the possibility that the combination of treatments used regulated the autophagy/cell proliferation interaction epigenetically. Furthermore, with respect to the genes that were evaluated, the predominant overexpression detected was that of *CD95L* and IL-8. Apoptosis can be induced by *CD95L* through its cognate receptor, *CD95*. In recent times, there has been further insight into the ability of *CD95L* to stimulate cell migration, differentiation, and proliferation [327]. It was discovered that EGFR transactivation by IL-8 stimulated cell proliferation in non-small cell lung cancer [328]. There is a significant degree of cross-talk between *HGFR* and *EGFR* [236]. Intense proliferation in the mOD HT29 cell group may be caused not only by overexpression of *CD95L* and IL-8 but also by *HGFR/EGFR* cross-signaling, according to these findings. By impeding *TLR9* and/or *HGFR* signaling pathways and

autophagy, respectively, modified tumor self-DNAs enable the development of novel anti-cancer therapies. Further research should be conducted to examine the impacts of concurrently administering these compounds to alternative tumor cell lines and animal models.

In the absence of stromal cells, HT29 cells have been shown to secrete small extracellular vesicle complexes resembling multivesicular bodies (MVBs) in recent research [329]. The ultrastructural changes observed in this investigation highlight the potential involvement of autophagy in either cellular protection or cell death promotion. The cell populations in which MVBs were identified exhibited upregulation of the *Beclin-1* and *PI3K* genes. This implies that, subsequent to the development of amphisomes, autolysosomal degradation may also occur via the interplay between the autophagosome and multivesicular body pathways [330]. The amphisome functions as a prelysosomal compartment that facilitates the convergence of the endocytic and autophagic pathways [331,332]. There are numerous possible outcomes for the contents of amphisomes, including extracellular release or lysosomal degradation. Both exosome biogenesis and autophagy are essential mechanisms for enhancing stress tolerance and maintaining cellular homeostasis [333]. Modulating these functions in cancer cells may reveal feasible therapeutic targets.

5.2 The interconnection of TLR9-mediated autophagy response and IGF1R signaling

By analyzing the proliferation and metabolic activity of HT29 colon cancer cells, we sought to determine how inhibition of IGF1R affects the way in which tumor-derived self-DNA influences TLR9 signaling and autophagy behavior.

Initially, we assessed the impact of TLR9 signaling modulation induced by self-DNA on the survival of HT29 cells helyett Initially, we evaluated the effect of self-DNA-induced TLR9 signaling modulation on the survival of HT29 cells. Beyond its application in cancer diagnostics, cfDNA has the potential to stimulate tumorigenesis and "genometastasis" as well as affect the immune response [334]. By activating signaling pathways through the interaction of cfDNA with specific cell receptors (including TLRs) or by increasing the transcriptional levels of multiple genes in a manner analogous to that observed with DNA aptamers, these biological effects can be induced [335].

In relation to HT29 cell proliferation and metabolic activity, we observed that gDNA treatment, with or without ODN2088, picropodophyllin, or chloroquine, had variable degrees of impact on cell survival. It is noteworthy that incubation solely with gDNA resulted in a reduction in the expression level of all genes examined (with the exception of TLR9). On the other hand, combining gDNA with inhibitors (specifically O, C, and PC) resulted in an increase in gene expression. This is partly explained by the survival-promoting and inhibitory effects of the gDNA-induced *TLR9* signaling pathway [293,298-302]. ODN2088 inhibited TLR9 signaling, resulting in an increase in cellular metabolic activity while not significantly affecting cell proliferation. Treatment with gDNA exclusively led to a significant reduction in cellular proliferation. In addition to gDNA-treated cells, ODN2088 exhibited a propensity to counteract the anti-proliferative effect of gDNA. This phenomenon may be attributed to variations in the expression levels of critical *TLR* signaling molecules, including *MAPK*, *PI3K*, or *NF-κB*, which may have significant functions [336].

The subsequent procedures involved examining the impact of alterations in the interplay between the *IGF1R* and *TLR9* signaling pathways on the survival of HT29 cells. Inhibition of IGF1R increased the metabolic activity of HT29 cells but had no discernible effect on proliferation, according to our findings. Treatment with gDNA (and its combination with O, P, C, and PC regimens) reduced cell division by a significant margin. Conversely, gDNA concurrently inhibited *IGF1R* and *TLR9* signaling (i.e., gOP), nullifying the suppression of cell proliferation. A noteworthy inconsistency was identified in the context of this matter: autophagy and apoptosis-related genes exhibited downregulation, whereas *Bcl-2* was found to be overexpressed. *Bcl-2* overexpression exerts anti-apoptotic and autophagy-suppressive effects, as evidenced by the increased number of cell divisions and decreased level of AVs in the gOP treatment group. In addition to regulating programmed cell death during tissue repair and development, *Bcl-2* may also possess oncogenic properties [337] by impeding cell proliferation rather than promoting it. CpG-ODN-induced TLR9 activation can increase IGF1 expression in intestinal epithelial cells [257]. Furthermore, intracellular IGF1 stimulates *Bcl-2* expression via the *IGF1R* and *EGFR* pathways [337]. Based on the findings, the combination of gDNA and picropodophyllin was able to inhibit the overexpression of *Bcl-2*. Nevertheless, the introduction of ODN2088 resulted in the upregulation of *Bcl-2*,

likely through a cross-talk between the *TLR9/EGFR* and *IGF1R/EGFR* signaling pathways [337,338]. Therefore, the concurrent application of IGF1R inhibitors and tumorous self-DNA may have anti-proliferative (therapeutic) implications. However, concurrent inhibition of TLR9 may negate this advantageous consequence (**Supplementary Figure 2**).

Additionally, we investigated the impact of the interaction between *TLR9* and *IGF1R* signaling on HT29 cell proliferation and autophagy. In this study, IGF1R inhibition decreased autophagy and mitigated the pro-autophagic effects of gDNA treatment and TLR9 signaling inhibition. In contrast, concomitant administration of chloroquine inhibited picropodophyllin, resulting in an increase in autophagy.

Prior research has demonstrated that cellular autophagy can occur through the inhibition of *PI3K/Akt*, leading to a subsequent downregulation of *AMPK/mTOR* [339,340]. It was observed that the administration of gDNA led to the suppression of these genes, ultimately resulting in a diminished capacity to inhibit autophagy initiation.

Activation of the IGF1R can stimulate the *PI3K/Akt* and *MAPK* signaling pathways, which are regulated by the phosphorylation of IRS1 and IRS2 subsequent to ligand binding [341,342]. The *PI3K/Akt* pathway activates the mTOR pathway, which regulates protein synthesis and cell proliferation via downstream effectors [342]. *AMPK* activity can be inhibited by IGF1R through the action of Akt1, which phosphorylates an AMPK inhibitory site [343] (**Supplementary Figure 2**). Recent research has identified picropodophyllin as an on-target, potent inducer of autophagic flux [283].

The experimental findings revealed that the introduction of picropodophyllin into gDNA-treated cells led to inhibitory effects on autophagy initiation and ultimately resulted in a reduced quantity of AVs, despite the relatively minor reductions in *PI3K*, *Akt*, *AMPK,* and *mTOR* gene expression. On the other hand, pharmacological inhibition of TLR9 resulted in the accumulation of AVs (compared to gP), suggesting that ODN2088 reduced the anti-autophagic effect of the gP combination. Considering that TLR9 maintains autophagic flux [344], this occurrence is also rational.

It has been discovered that chloroquine inhibits cell proliferation by impeding late-stage autophagy and inducing apoptosis mediated by mitochondria [345]. Although the gP combination exhibited anti-autophagic properties, the introduction of chloroquine (referred to as gPC) led to a substantial upregulation of autophagy and a reduction in cell

proliferation. Based on these, we think that the complex balance between the factors that stop and start autophagy has a major impact on the final biological outcome. The different effects of P, O, and C treatments on HT29 cell proliferation and autophagy, both alone and together, show that the IGF1R-related and unrelated autophagy machinery have a "Janus-faced" effect on cell proliferation, in addition to the *TLR9-IGF1R-Bcl-2* molecular link.

We also examined what happened to the HT29 stem-like phenotype when autophagy and IGF1R were inhibited. High levels of autophagy and low levels of cell proliferation were seen in the groups that were given inhibitors (gP, gC, gO, and gPC). These groups also had CD133-positive cells. This shows that blocking *IGF1R* and/or autophagy may be therapeutically ineffective. Unbalanced *IGF1R* signaling has the potential to stimulate cancer cell proliferation and initiate cancer reprogramming in various tumor tissues [250,346]. It has been demonstrated recently that *IGF1R* facilitates epithelial-mesenchymal transition and cancer stem cell properties by activating *Akt* [347,348]. Moreover, various cell types recognize autophagy for its role in promoting stem cell viability [349]. When IGF1R is inhibited, protective autophagy may be induced concurrently, which may stimulate cell proliferation and inhibit apoptosis. Thus, it counteracts its own initial biological effects on cells via autophagy [236]. The combination of IGF1R inhibition and autophagy-disruptive agents has the potential to impede autophagy, thereby impeding the proliferation of cancer cells and augmenting apoptosis [236]. The autophagy process in CD133-positive HT29 stem cells could not be detected because these cells were scattered in the cell smear and could not be distinguished on TEM sections. The only information available is that the HT29 treatment groups have an autophagy flux that promotes the development of the CD133 phenotype. Notwithstanding this, future research should undoubtedly focus on evaluating the autophagic flux within HT29 stem-like cells that express CD133. Based on the results of our experiments, it is likely that autophagy induced by different combinations of gDNA and inhibitors used cannot prevent HT29 cell death. Additionally, it may compel stem-like HT29 cells that are positive for CD133 to promote their own survival. Curcumin was discovered to promote the proliferation and autophagic survival of colon cancer stem cells [63], providing support for this hypothesis. This discovery indicates that autophagy

confers a survival advantage, allowing colorectal cancer progenitor cells to persist indefinitely [351].

We investigated the correlation between autophagy and IGF1R inhibition in relation to ultrastructural alterations using TEM. MVB signals were identified in HT29 cells that were treated with gP. At least three potential causes can be identified for this phenomenon. The first is that receptors (such as the receptor tyrosine kinase IGF1R) can be rapidly recycled back to the plasma membrane via "back fusion" from early endosomes. As luminal acidification increases during the maturation of the early endosome, its biochemical composition alters. MVBs, or intraluminal vesicles, are ultimately produced [348]. *IGF1R* can be delivered for recycling at this stage. Second, endocytosed IGF1R-containing MVBs are capable of fusing with the plasma membrane and releasing their cargo as exosomes. Reports indicate that cells secrete IGF1R in this manner [250,347,348]. Thirdly, recent research has shown that HT29 cells are capable of releasing MVB-like small extracellular vesicle complexes [349] in the absence of stromal cells. The observed ultrastructural changes in our research highlight the potential involvement of autophagy in either cellular protection or the induction of cell death. The gP cell group, which exhibited the presence of MVBs alongside autophagic vacuoles, demonstrated an upregulation of autophagy-related protein expression (*Beclin-1*, *ATG16L1*, *LC3B*) in comparison to the non-treated control cells. Based on the findings, it is probable that autolysosomal degradation occurs subsequent to the development of amphisomes through the interplay between the autophagosome and multivesicular body pathways [350]. The amphisome serves as a junction between the endocytic and autophagic pathways [351,352], operating as a prelysosomal compartment. There are numerous possible outcomes for the contents of amphisomes, including extracellular release or lysosomal degradation. Both exosome biogenesis and autophagy are essential mechanisms for enhancing stress tolerance and maintaining cellular homeostasis [329-331,353,354]. Modulating these functions in cancer cells may reveal feasible therapeutic targets.

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6. CONCLUSIONS

In conclusion, the purpose of this research was to determine how modified tumorous self-DNA treatments affected the survival and proliferation of HT29 colon cancer cells through the intricate interplay of *HGFR/IGF1R*, *TLR9* signaling, and autophagy inhibition.

Concerning the HGFR inhibitory experiments, we have demonstrated that the nature of the DNA modification influences the reduction in cell proliferation. By employing TLR9 blocking, this effect has been reversed. A marginal increase in *MyD88* expression was observed in response to self-DNA treatments. HT29 cell proliferation was observed to be augmented in conjunction with the upregulation of *MyD88*. Similarly, caspase-3 expression exhibited alterations consistent with those of *MyD88*. Therefore, incubation with modified self-DNAs was able to inhibit apoptosis-induced proliferation of compensating HT29 cells. All forms of self-DNA modification upregulate autophagy-related gene expression. DISU inhibited the proliferation-reducing effects of genomic and hypermethylated DNAs, whereas fragmented DNA showed the opposite effect. The most significant inhibition of cellular proliferation was observed when gDNA, DISU, and chloroquine were utilized concurrently. The inhibitory effect of *LC3B* may have compensated for the proliferation-stimulating effect of *STAT3* overexpression in this instance, suggesting that the *HGFR* inhibitor-mediated autophagy process involves the *HGFR-mTOR-ULK1* molecular cascade. The maximum degree of cellular proliferation was detected upon the simultaneous administration of mDNA, ODN2088, and DISU. In this case, we saw that autophagy-related genes and both canonical and non-canonical *HGFR* signaling pathways were less expressed. In addition, the observed ultrastructural alterations provide further evidence for the context-dependent function of autophagy and HGFR inhibition on cell survival and proliferation.

Regarding the studies into the effects of IGF1R inhibition, it was found that giving tumorous self-DNA, picropodophyllin, HT29 cells, or chloroquine together changes metabolic activity and proliferation in different ways. Inhibiting *TLR9* signaling negatively impacts the effect of self-DNA treatment on cell proliferation. The concurrent administration of *IGF1R* inhibitors and tumor-derived self-DNA exhibits anti-proliferative properties. However, concomitant inhibition of *TLR9* signaling undermines this advantageous effect. It has been observed that the intricate equilibrium between

inhibitory and promotional factors in autophagy significantly influences its ultimate intensity and biological consequences. The distinct impacts of picropodophyllin, ODN2088, and chloroquine, either individually or in combination, on HT29 cell proliferation and autophagy indicate that the *IGF1R*-associated and non-*IGF1R*-associated autophagy machinery exhibit a "Janus-faced" nature with regard to their influence on cell proliferation, in addition to the *TLR9-IGF1R-Bcl-2* molecular linkage. Autophagy induced by various combinations of tumorous self-DNA and inhibitors may not be adequate to prevent the irreversible demise of HT29 cells; however, it may enable the survival of certain CD133-positive stem-like cancer cells, thereby promoting the recurrence of colorectal cancer. We also observed ultrastructural alterations that support the notion that autophagy and *IGF1R* inhibition influence cell survival and proliferation in a context-dependent manner.

Inhibitors that target autophagy, *TLR9*, *HGFR*/*IGF1R,* and/or autophagy signaling in combination would be instrumental in the advancement of personalized anti-tumor treatments. Nonetheless, further TLR9-expressing cell lines should be utilized to validate our current investigations. Additional research is required to examine the biological effects of self-DNA fragments that have been modified, as factors such as fragment length or methylation status may impact the results of the experiments.

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7. SUMMARY

Self-DNA-induced TLR9 signaling and autophagy response in HT29 colon cancer cells were closely related, affecting cell survival and differentiation. Interfering with *HGFR* hinders autophagy and promotes colorectal cancer. *IGF1R* activation drives colorectal cancer development and progression. Debate surrounds the effect of IGF1R inhibition on autophagy. The exact methods by which *HGFR* or *IGF1R* suppression affects *TLR9*/autophagy signaling in HT29 cancer cells are unclear. We investigated how suppressing *HGFR* or *IGF1R* impacts *TLR9*-autophagy signaling in HT29 cells. We examined how these components interact by measuring cell proliferation, metabolism, TLR9, HGFR, IGF1R, and autophagy inhibitory tests. Gene expression, immunocytochemistry, transmission electron microscopy and WES Simple autophagy flux measurements were also examined.

In HGFR inhibitory tests, we found that *MyD88* and *caspase-3* promoted HT29 cell growth. Incubation with self-DNAs may decrease apoptosis-induced compensatory cell growth. HGFR inhibition blocks the proliferation-reducing impact of genomic and hypermethylated DNA, while fragmented DNA is unaffected. Chloroquine, HGFR inhibitor, and genomic DNA showed the least cell proliferation. The *HGFR-mTOR-ULK1* molecular cascade may be implicated in HGFR inhibitor-mediated autophagy since *LC3B* inhibited *STAT3* overexpression and reversed its proliferation-stimulating action. When given together, hypermethylated DNA, TLR9, and HGFR inhibitors increased cell proliferation the most. Autophagy-related genes and conventional and non-canonical *HGFR* signaling pathways were downregulated. Ultrastructural alterations support the context-dependent effect of HGFR inhibition and autophagy on cell survival and proliferation. In IGF1R inhibitory assays, tumor-derived self-DNA and IGF1R inhibitors have anti-proliferative potential. *TLR9* signaling inhibition reverses this effect. Picropodophyllin, ODN2088, and chloroquine all had different effects on HT29 cell proliferation and autophagy. This suggests that autophagy mechanisms have "Janus-faced" effects on cell proliferation, depending on whether they are linked to or not linked to the *IGF1R*. Self-DNA and inhibitors do not promote autophagy, but they let CD133-positive stem-like HT29 cells survive. In the development of more individualized anti-tumor therapies for colorectal cancer, the discovery of novel forms of combined inhibitors of *HGFR* or *IGF1R*, autophagy, and/or *TLR9* signaling would be instrumental.

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9. BIBLIOGRAPHY OF THE CANDIDATE'S PUBLICATIONS

Cumulative IF: 16.5

Publications / abstracts related to PhD dissertation (subtotal IF: 11.2)

1. **Bohusné Barta Bettina**, Simon Ágnes, Nagy Lőrinc, Dankó Titanilla, Raffay Regina Eszter, Petővári Gábor, Zsiros Viktória, Sebestyén Anna, Sipos Ferenc, Műzes Györgyi. Survival of HT29 cancer cells is influenced by hepatocyte growth factor receptor inhibition through modulation of self-DNA-triggered TLR9-dependent autophagy response. PLOS ONE 2022; 17(5): e0268217. IF: 3.7 (Q1)

2. Sipos Ferenc / **Bohusné Barta Bettina**, Simon Ágnes, Nagy Lőrinc, Dankó Titanilla, Raffay Regina Eszter, Petővári Gábor, Zsiros Viktória, Wichmann Barnabás, Sebestyén Anna, Műzes Györgyi. Survival of HT29 cancer cells is affected by IGF1R inhibition via modulation of self-DNA-triggered TLR9 signaling and the autophagy response. PATHOLOGY AND ONCOLOGY RESEARCH 2022; 28: 1610322. IF: 2.8 (Q2)

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1. Műzes Györgyi, **Bohusné Barta Bettina**, Sipos Ferenc. Colitis and Colorectal Carcinogenesis: The Focus on Isolated Lymphoid Follicles. BIOMEDICINES 2022; 10(2): 226. IF: 4.7 (Q1)

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11. SUPPLEMENTS

SUPPLEMENTARY TABLE 1 | Assayed genes with probe NSIDs in NanoString experiments.

***TLR9*-signaling and *NF-κβ* activation:** *TLR9* (Toll-like receptor 9; NM_017442.2:985), *MyD88* (Myeloid differentiation factor 88; NM_002468.3:2145), *IRAK2* (Interleukin 1 receptor associated kinase 2; NM_001570.3:1285), *TRAF6* (Tumor necrosis factor receptor associated factor 6; NM_145803.2:745), IL1-β (Interleukin 1β; NM_000576.2:840), IL-8 (Interleukin 8; NM_000584.2:25), *NF-κB* (Nuclear factor- κB; NM_003998.2:1675).

Extrinsic and intrinsic apoptosis-related genes: *CD95* (Fas; NM_152876.1:1740), *CD95L* (Fas-ligand; NM_000639.1:625), *Cytochrom-c* (NM_001916.4:344), *Caspase-3* (NM_004346.3:2156), *Caspase-8* (NM_033355), *Caspase-9* (NM_032996).

Anti-apoptotic and autophagy suppressor genes: *PI3KCA* (Phosphoinositide 3-kinase; NM_006218.2:2445), *Akt* (Ak strain transforming; NM_001014432.1:1275), *mTOR* (Mechanistic/mammalian target of rapamycin; NM_004958.3:1865), *Bcl-2* (B-cell lymphoma 2; NM_000657.2:5).

Pro-apoptotic and autophagy activator genes: *MAPK* (Mitogen-activated protein kinase; NM_002755.2:970), *AMPK* (AMP-activated protein kinase; NM_006251.5:366), *Bax* (BCL2 associated X; NM_138761.3:342).

Autophagy genes: *Beclin-1* (NM_003766.2:810), *ATG16L1* (Autophagy related 16 like 1; NM_017974.3:2405), *MAP1LC3B* (Microtubule-associated proteins 1A/1B light chain 3B; NM_022818.4:1685), *ULK1* (Unc-51 like autophagy activating kinase; NM_003565.1:465), *Ambra-1* (activating molecule in Beclin-1-regulated autophagy; NM_017749).

***c-Met/HGFR* and c-*Met* canonical and non-canonical signaling pathways:** *HGFR* (NM_001127500.1:1925), *PI3KCA* (see above), *STAT3* (Signal transducer and activator of transcription 3; NM_003150.3:2060), *CD95* (see above).

***IGF1R* signaling pathway:** *IGF1R* (Insulin-like growth factor 1 receptor; NM_000875); *MAPK* (Mitogen-activated protein kinase; NM_002755.2:970); *PI3K* (Phosphoinositide 3-kinase; NM_006218.2:2445); *Akt* (Ak strain transforming; NM_001014432.1:1275).

HT29 cancer cell stemness-related gene: *CD133* (NM_006017).

Housekeeping genes: *C1orf43* (NM_015449.2:477), *CHMP2A* (NM_014453.3:241), *PSMB2* (NM_002794.3:639), *RAB7A* (NM_004637.5:277), *REEP5* (NM_005669.4:280), *SNRPD3* (NM_004175.3:309), *VCP* (NM_007126.2:615), *VPS29* (NM_016226.4:565).

SUPPLEMENTARY TABLE 2 | Standard deviation results of the Taqman fold changes in HGFR studies [248]

The table displays the related standard deviation results (SD) ($p<0.05$; n=3).

g/f/m: genomic/fragmented/hypermethylated DNA; K: control; O: ODN2088 CpG oligonucleotide; D: DISU; C: chloroquine

SD values	*TLR9*	*mTOR*	*ATG16L1*	*LC3B*	*BECN1*	*HGFR*	*PI3KCA*	*STAT3*	*CD95*
K	0.12	0.23	0.18	0.24	0.29	0.16	0.13	0.22	0.28
Kg	0.13	0.22	0.19	0.34	0.19	0.24	0.19	0.18	0.17
Kf	0.22	0.22	0.25	0.19	0.31	0.21	0.24	0.18	0.25
Km	0.21	0.27	0.28	0.29	0.32	0.28	0.21	0.27	0.28
gO	0.27	0.29	0.29	0.21	0.27	0.21	0.27	0.32	0.29
gD	0.18	0.21	0.24	0.19	0.31	0.19	0.18	0.21	0.24
gC	0.32	0.29	0.32	0.24	0.25	0.22	0.32	0.29	0.32
gOD	0.12	0.23	0.18	0.37	0.29	0.16	0.12	0.23	0.18
gOC	0.19	0.21	0.19	0.34	0.19	0.24	0.29	0.19	0.19
gDC	0.22	0.15	0.25	0.19	0.31	0.21	0.22	0.32	0.23
fO	0.21	0.27	0.28	0.29	0.22	0.28	0.21	0.27	0.28
fD	0.27	0,29	0.29	0.21	0.27	0.21	0.27	0.29	0.29
fC	0.18	0.21	0.24	0.19	0.31	0.19	0.18	0.21	0.24
fOD	0.32	0.29	0.32	0.34	0.25	0.22	0.32	0.29	0.32
fOC	0.12	0.23	0.18	0.24	0.39	0.16	0.12	0.23	0.18
fDC	0.19	0.19	0.19	0.34	0.19	0.24	0.19	0.19	0.19
mO	0.22	0.22	0.25	0.19	0.31	0.21	0.23	0.22	0.25
mD	0.21	0.27	0.28	0.36	0.32	0.28	0.21	0.27	0.28
mC	0.27	0.29	0.29	0.21	0.37	0.29	0.27	0.29	0.19
mOD	0.28	0.31	0.24	0.19	0.31	0.19	0.18	0.24	0.24
mOC	0.32	0.29	0.32	0.24	0.25	0.22	0.32	0.29	0.32
mDC	0.12	0.25	0.18	0.24	0.29	0.16	0.14	0.23	0.18

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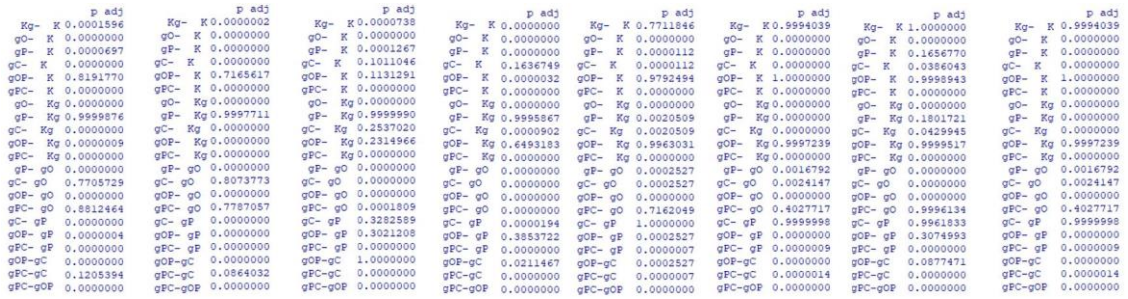
SUPPLEMENTARY TABLE 3 | Standard deviation results of the Taqman fold changes in IGF1R studies [276]

The table indicates the SD values in each case ($p<0.05$; n=3).

g: genomic DNA; K: control; O: ODN2088 CpG oligonucleotide; P: picropodophyllin; C: chloroquine

SD values	*mTOR*	*ATG16L1*	*LC3B*	*BECN1*	*IGF1R*	*TLR9*
K	0.12	0.23	0.18	0.24	0.29	0.16
gOP	0.19	0.19	0.19	0.34	0.19	0.24
gP	0.22	0.22	0.25	0.19	0.31	0.21
Kg	0.21	0.27	0.28	0.29	0.32	0.28
gC	0.27	0.29	0.29	0.21	0.27	0.21
gO	0.18	0.21	0.24	0.19	0.31	0.19
gPC	0.32	0.29	0.32	0.24	0.25	0.22



SUPPLEMENTARY FIGURE 1 | Regarding immunocytochemistries, the results of the Tukey HSD test and the adjusted p-values of the comparisons of each treatment group can be seen ($p<0.05$ represents statistical significance) [276]

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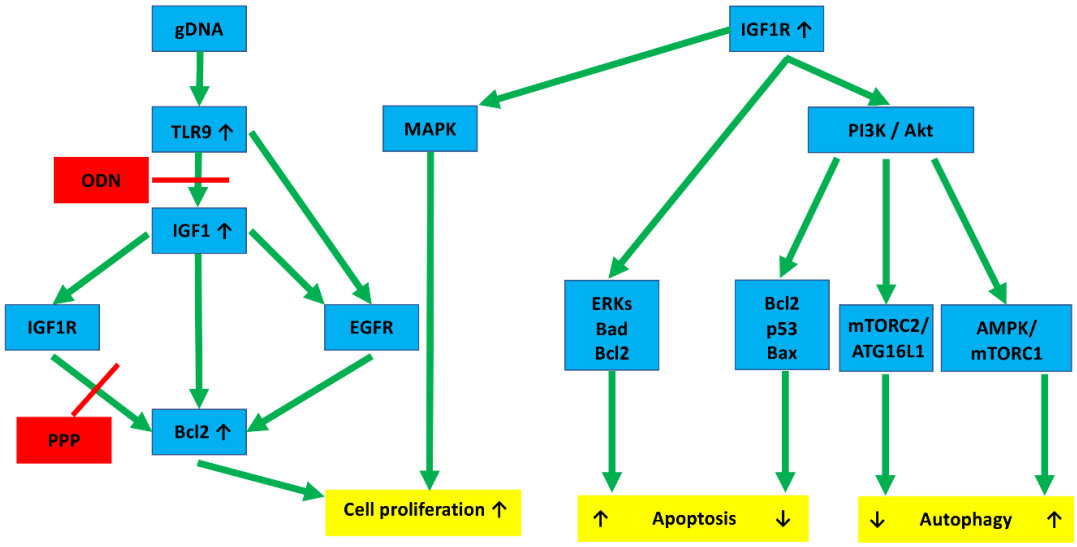

SUPPLEMENTARY FIGURE 2 | Hypothesized molecular links connecting IGF1R and TLR9 signaling to autophagy and cell proliferation in HT29 cancer cells [276]

TLR9 binding of gDNA through IGF1 and IGF1R activation promotes cell division by enhancing Bcl2. ODN and PPP may inhibit this, but the inhibitory effect can be counteracted by EGFR cross-activation. IGF1R activation via the PI3K/Akt pathway affects autophagy. If it is through the AMPK/mTORC1 pathway, it is a stimulant. If it is through the mTORC2/ATG16L1 pathway, it is primarily inhibitory. Similarly, IGF1R inhibits apoptosis via the Akt-Bcl2-p53-Bax proteins, whereas the Erk-Bad-Bcl2 pathway tends to stimulate it. The final effects are always context-dependent.

Red lines: inhibitory effect; gDNA: genomic self-DNA; TLR9: Toll-like receptor 9; ODN: oligodeoxynucleic acid 2088; IGF1: insulin-like growth factor 1; IGF1R: insulin-like growth factor 1 receptor; EGFR: epidermal growth factor receptor; PPP: picropodophyllin; Bcl2: B-cell lymphoma 2; MAPK: mitogen-activated protein kinase; PI3K: phosphoinositide 3-kinase; Akt: Ak strain transforming; Bax: BCL2 associated X; AMPK: AMP-activated protein kinase; mTOR: mammalian target of rapamycin; mTORC1/2: mTOR complex 1/2; ERKs: extracellular signal-regulated kinases; ATG16L1: Autophagy Related 16 Like 1; Bad: BCL2-associated agonist of cell death