UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE BIOLOGIA VEGETAL

Regulation of LDLR expression in breast cancer cells and its implication in tumour progression

Pedro Miguel da Silva Marreiros

Mestrado em Biologia Molecular e Genética

Dissertação orientada por:
Ana Magalhães
Carla Lopes

2021

Acknowledgements

Gostaria de agradecer à Faculdade de Ciências da Universidade de Lisboa e ao grupo de docentes de Biologia Molecular e Genética pela formação que me foi dada, não só a mim, mas a todos os meus colegas ao longo do mestrado e também ao Instituto de Medicina Molecular por permitir que eu terminasse o meu último ano de mestrado aqui a realizar a tese.

Ao laboratório SDias e toda a sua equipa de investigação por me acolherem com muito carinho e por me terem tratado incrivelmente bem neste último ano. Estiveram sempre lá para me ajudar quando estava com dificuldades e graças a eles consegui aprender imensa coisa neste meu percurso até chegar aqui.

Um especial agradecimento à minha orientadora, Doutora Ana Magalhães, por toda a ajuda e enorme disponibilidade e acompanhamento que me foi dada durante ao longo deste ano. Esteve sempre lá para mim quando mais precisei e sem ela não seria possível alcançar mais este objetivo.

Quero agradecer aos meus colegas de curso e aos meus amigos por me terem acompanhado neste percurso e por me ajudarem a cumprir esta etapa da minha vida.

Por fim, agradeço imenso à minha família, especialmente aos meus pais, e à minha namorada por me manterem sempre motivado e por me incentivarem a nunca desistir nos momentos mais difíceis.

Resumo

A progressão tumoral é um fenómeno complexo influenciado por vários fatores intrínsecos e extrínsecos às próprias células tumorais. Uma característica particular das células tumorais é o facto de terem a capacidade de alterar o seu metabolismo, de modo a sustentar a sua elevada proliferação. O colesterol é um componente essencial da membrana citoplasmática e, como tal, essencial à proliferação celular, sendo que a maioria das células obtém o colesterol de que necessita através da internalização de lipoproteínas de baixa densidade (LDL). A internalização do LDL é feita principalmente por meio de recetores de membrana tais como o recetor de LDL (LDLR). É hoje sabido que alterações ao nível do metabolismo do colesterol, quer a nível celular ou sistémico afeta a progressão tumoral. Em particular, a sobre expressão de LDLR em células de cancro da mama foi recentemente associada a um aumento da progressão tumoral e a um pior prognóstico. Os fatores do microambiente tumoral que poderão levar a um aumento de expressão do LDLR em células de cancro da mama, são, no entanto, desconhecidos. Os mecanismos que regulam a expressão do LDLR foram extensivamente estudados em hepatócitos e fibroblastos, bem como noutras células como as células mesangiais do rim. Neste contexto, foi identificado um mecanismo de loop de feedback negativo pelos níveis de colesterol intracelular bem como mecanismos independentes de esteróis, tais como a sinalização por citocinas e fatores de crescimento. No entanto, a regulação da expressão do LDLR em células do cancro da mama foi, até agora, pouco estudada e a identidade de fatores que podem levar a um aumento da expressão do LDLR nas células tumorais de cancro da mama é maioritariamente desconhecida. O presente trabalho tem, então, como principal objetivo colmatar estas lacunas. Por um lado, estudou-se a regulação da expressão de LDLR pelo colesterol, com o intuito de verificar se o mecanismo de feedback negativo se mantém nestas células, apesar de transformadas. Por outro lado, analisou-se a capacidade de citocinas presentes no microambiente tumoral contribuírem para a regulação da expressão do LDLR. Para tal, foram utilizadas três linhas celulares de cancro da mama suscetíveis a alterações no metabolismo do colesterol: as linhas celulares humanas MDA-231 e MCF-7 e a linha celular de ratinho 4T1. Quanto à regulação da expressão do LDLR pelo colesterol, verificou-se que tanto na linha celular MDA-231, quanto na linha celular MCF-7, existe um mecanismo de feedback negativo. Quanto às citocinas, identificou-se o IFNγ como sendo capaz de induzir um aumento da expressão do LDLR ao nível da proteína, em todas as linhas celulares estudadas. Desta forma, posteriormente, analisou-se a consequência de tal aumento de expressão, bem como o mecanismo através do qual o IFNγ leva a um aumento de expressão do LDLR – estes estudos foram feitos na linha celular MDA-231. Observou-se, então, que o aumento da expressão do LDLR pelo IFNγ levou a um aumento da internalização de colesterol pelas células, uma vez que se observou um aumento de colesterol em células tratadas com IFNγ e LDL quando comparado com células tratadas só com LDL. Mais ainda, este aumento de colesterol é revertido quando a entrada de LDL nas células através do LDLR é inibida por via de um anticorpo específico. Ao nível funcional, podemos verificar que células de cancro da mama expostas simultaneamente a LDL e a IFNγ proliferam tanto como células só expostas a LDL; e mais do que células apenas expostas a IFNγ. Uma vez que o IFNγ, por si só, diminui a proliferação das células tumorais, deduzimos que o efeito na presença de ambas as moléculas se deva ao efeito do IFNγ na internalização de LDL por parte das células tumorais. No entanto, uma experiência com o bloqueio do LDLR teria que ser efetuada para comprovar se esse efeito se deve, efetivamente, ao aumento da expressão de LDLR. Do ponto de vista da migração, uma vez que tanto o LDL como o IFNγ provocam um aumento da capacidade migratória destas células,

nenhuma conclusão maior pode ser retirada. Do ponto de vista mecanístico, verificou-se que o IFNγ está a atuar nestas células essencialmente ao nível da via JAK / STAT1 que influencia a expressão do LDLR. Mais ainda, os nossos dados sugerem que o IFNγ está a influenciar a expressão do LDLR ao nível da transcrição do gene. *In vivo*, fomos capazes de demonstrar, em tumores de ratinho, que a localização do LDLR é heterogénia, seria interessante no futuro verificar se as zonas com maior expressão estão associadas a zonas de exposição elevada a IFNγ. Tomados em conjunto, os dados presentes neste trabalho contribuem para uma melhor compreensão de como a internalização do colesterol é regulada em células do cancro da mama, bem como as suas consequências para a progressão tumoral. Mais ainda, acresce com conhecimento novo sobre a ação das citocinas imunomoduladoras presentes no microambiente tumoral. Acreditamos que este conhecimento venha a contribuir para melhorar a capacidade de prevenir, diagnosticar e combater o cancro da mama.

Palavras-chave: LDLR, IFNγ, MDA-231, cancro da mama, colesterol, citocinas, microambiente tumoral

Abstract

Tumour progression is a complex process that is influenced by both tumour cell intrinsic and extrinsic factors. A particular feature of cancer cells is the ability to alter their metabolism in order to sustain proliferation. Cholesterol is an essential component of cellular membranes and as such essential for proliferative cells and cells all over the body acquire the cholesterol they need mainly from the circulating low-density lipoprotein (LDL). The internalization of LDL is mostly done through membrane receptors such as the LDL receptor (LDLR). Alterations at the level of cellular or systemic cholesterol metabolism have been shown to influence tumour progression. LDLR overexpression, in particular, was recently associated with breast cancer progression and worse prognostic. The most studied and best known way of regulating LDLR expression in cells is through a negative feedback loop induced by intracellular cholesterol levels. This has been mainly shown in hepatocytes and fibroblasts and also in other cell types such as smooth vascular muscle cells, liver cells and mesangial cells. Sterol-independent regulation of LDLR expression, including trough cytokines has also been described in non-malignant cells. Whether breast cancer cells maintain an intact negative feedback loop when exposed to external cholesterol and whether other factors apart from cholesterol regulate LDLR expression in such cells is mainly unknown. The present project aims at analysing the regulation of LDLR by LDL on breast cancer cells and also on testing the hypothesis that there are, at the tumour microenvironment, cytokines able of inducing LDLR expression on breast cancer cells. In order to detect differences in LDLR expression, three breast cancer cell lines susceptible to changes in cholesterol metabolism were used, the MDA-231 and MCF-7 human cell lines and the 4T1 mouse cell line. First of all, we were able to show that both the MDA-231 and the MCF-7 cell line downregulate its LDLR expression when in the presence of high LDL concentrations in the media, demonstrating that they have a functional negative feedback loop mechanism for cholesterol homeostasis. Moreover, we were able to show that IFNγ induces LDLR expression in all cell lines studied. Therefore, this cytokine was chosen for the consequent experiments, which were then performed only on the MDA-231 cell line. Mechanistically, our data suggest that IFNγ is signalling on these cells mainly through the JACK/STAT1 pathway and that inhibiting STAT1 phosphorylation rescues the effect of IFNγ on LDLR expression. Functionally, we show that the increase in LDLR expression by IFNγ led to an increase in the cholesterol uptake in the cells and it seems to induce resistance to IFNγ-induced proliferation arrest. Taken together, we believe that our data will contribute to further characterize how cholesterol metabolism is regulated in breast cancer cells and also increase our knowledge about the action of TME immunomodulatory cytokines, leading to potential new strategies for better preventing, predicting and treating breast cancer in patients.

Keywords: LDLR, IFNγ, MDA-231, breast cancer, cholesterol, cytokines, tumour microenvironment

Table of Contents

Figures Table

Abbreviations

LDLR	Low-Density Lipoprotein receptor
LDL	Low-Density Lipoprotein
TME	Tumour microenvironment
BC	Breast Cancer
SREBP2	Sterol regulatory element-binding protein-2
IFNγ	Interferon-gamma
PCSK9	Proprotein convertase subtilisin/Kexin type 9
TNFα	Tumour necrosis factor alpha
TGFβ	Growth factor beta transforming factor
IL-1β	Interleukin-1β
IL10	Interleukin-10
FBS	Fetal bovine serum

Introduction

Cancer Progression

Cancer is a very large group of diseases that have in common the development of abnormal cells. 80% of cancers arise in the epithelial tissue and are called carcinomas. Very often carcinomas metastasize, meaning that cancer cells originating from one tissue, disseminate and start growing in a different organ. Metastases are responsible for most deaths in cancer. Cancer progression refers to a series of events, that may happen sequentially or in parallel, occurring at primary tumours, frequently culminating in metastases. In the beginning, for very different reasons, genetic alterations in the genome of epithelial cells occur and cells start to divide and survive without control. When the tumour reaches a certain size, it needs more and more nutrients and gradually gets less oxygen from blood vessels in its environment – creating hypoxic regions. Hypoxia, in turn, induces the production of factors that lead to the formation of new blood vessels from existing ones (angiogenesis). When tumours are malignant, they tend to invade and travel to other tissues. The cells first start to invade the surrounding tissue, eventually migrate, and enter circulation, as they loose characteristics of adhesive epithelial cells and gain migratory properties of mesenchymal cells (Epithelial-Mesenchymal Transition). Cancer cells travel through the bloodstream and at a certain point extravasate into secondary tissue where it is believed they go through the opposite process, Mesenchymal-epithelial transition (MET) and thus proliferating again and forming a secondary tumour, in other words, a metastasis [1]. In breast cancer, most deaths occur due to metastases. In Portugal, approximately 6000 new cases of breast cancer are detected annually, and 1500 women die as a result of this disease [2]. Breast cancer became the most common cancer globally as of 2021, accounting for 12% of all new annual cancer cases worldwide, according to the World Health Organization [3]. Worldwide, breast cancer is the main type of cancer in women, representing about 25% of all cases. It is a disease that appears more in developed countries and, in 2018, 2 million new cases were diagnosed, and 627,000 deaths were reported [4]. A big effort is being taken to identify the cellular and molecular mechanisms that regulate cancer progression and in designing new, predictive, preventive, and curative strategies concerning this life-threatening disease.

Altered tumour cell metabolism, a hallmark of tumour progression

Among many factors known to contribute to tumour progression, the reconfiguration of cell metabolism has been recently considered as one of its hallmarks. In normal cells, under aerobic conditions, glucose is processed through glycolysis, followed by oxidative phosphorylation in mitochondria. However, under anaerobic conditions, glycolysis is favoured and there is almost no action of oxygen-consuming mitochondria. In cancer cells it was first observed by Otto Warburg that even in the presence of oxygen, cells reprogrammed their glucose metabolism, limiting their energy metabolism mainly to glycolysis, leading to a state called aerobic glycolysis [5-7]. This reprogramming of energy metabolism is counterintuitive, as it is the oxidative phosphorylation that provides more energy in form of ATP to cells. However, according to a long-forgotten hypothesis [8] and recently brought back [9], increased glycolysis results in a greater shift of glycolytic intermediates to various biosynthetic pathways, thus facilitating the biosynthesis of macromolecules and organelles necessary for the formation and development of new cells. Furthermore, it was found that certain tumours have two subpopulations of cells that

differ in their metabolic pathways, one consisting of glucose-dependent cells, Warburg effect, thus producing lactate, while cells from the other subpopulation use the lactate produced by their neighbours as its main source of energy. Thus, these two populations form a type of symbiosis, a similar process of muscle function [10-12]. As for lipid metabolism, most human cells use circulating lipids for the synthesis of other structural compounds, such as cholesterol, fatty acids, phospholipids, sphingolipids, and isoprenoids. However, cancer cells have a higher rate of fatty acid synthesis, as an important source of energy via β-oxidation or for storage in the form of triglycerides, or even for conversion into phospholipids for cell membrane production [13,14]. The altered metabolism is proving to be as pervasive in cancer cells as many of the other cancer-associated characteristics, therefore, this has been accepted as an emerging cancer hallmark [15].

Cholesterol Metabolism and Cancer

Cholesterol is an essential component of cellular membranes and a precursor of several hormones. As part of cell membranes, it also contributes to signal transduction and membrane trafficking, particularly endocytosis. Due to its amphipathic nature, cholesterol is insoluble in the plasma and cannot be transported freely in the bloodstream. Because of this, cholesterol and other lipids travel within low-density lipoproteins (LDL) or high-density lipoproteins (HDL). High plasma LDL levels are a well-known risk factor for many diseases such as atherosclerosis and other cardiac diseases, mainly, due to its deposition in blood vessel walls, which restricts blood flow [16]. In cancer, obesity and dyslipidaemia have been associated with a possible increase in the probability of developing the disease [17]. However, few studies have been done to try to find the cellular and molecular mechanisms linking the overabundance of lipids and the behaviour of the tumour. In breast cancer cells cultured *in vitro*, it was found that exposure to LDL induces aggressive behaviours, such as increased cell proliferation, migration, and loss of adhesion. It has been suggested that the proliferative effect induced by LDL may be dependent on the activation of the Akt and ERK pathways and that the loss of adhesion and increased migration could be explained due to a decreased expression of adhesion molecules, such as related family member to cadherin 3, CD226, Claudin 7 and Occludin. More frequent metastasis was also observed in mice with breast cancer when fed on a high cholesterol diet that increases plasma LDL levels [18-22].

The LDL-Receptor Function, Structure and Regulation

The majority of LDL absorption by cells is mediated by the LDL receptor (LDLR), a transmembrane protein that captures LDL from the extracellular space and is then internalized in endocytic vesicles to transport LDL into the intracellular space so that it can be digested, while LDLR is recycled and returns to the cell surface [23]. The human LDLR is encoded by a gene of approximately 45 kb located on chromosome 19, which will later be translated into a protein of 860 amino acids [24]. LDLR has five functionally distinct regions, a region of binding to the N-terminal ligand where LDL will bind, an epidermal growth factor (EGF) -precursor homology region that regulates the dissociation of LDL to the receptor within the cell, a region containing O-linked sugars where the sugars bind giving greater support to the receptor, a transmembrane domain that anchors the protein to the membrane and finally a C-terminal cytosolic domain that is involved in endocytosis and intracellular transport [23].

LDLR levels are maintained by multiple regulatory mechanisms: At the transcriptional level, LDLR gene expression is regulated through negative feedback due to the content of cholesterol

present in the cells and plasma. When cells are low in cholesterol, the LDLR gene is actively transcribed and thus cholesterol-rich lipoproteins will be quickly internalized. Inversely, when cholesterol accumulates in cells, the LDL receptor gene is repressed, [25,26]. Mechanistically LDLR gene expression is highly regulated by a sterol regulatory element-binding protein-2 (SREBP2), the first sterol-regulated transcription factor described, in normal cells of the human body. SREBP2 is a transmembrane protein of the endoplasmic reticulum that is in an inactive state before being transported to the Golgi complex where it will be cleaved by an SREBP cleavage-activating protein (SCAP), which depends on low cholesterol levels to be able to cleave SREBP2. This will lead to the activation of the transcription factor SREBP-2 and its translocation to the nucleus, promoting the transcription of the LDLR gene [27]. SREBPs also regulate the transcription of several genes involved in cholesterol synthesis and fatty acid synthesis/absorption, coordinating the two main building pillars of cell membranes [28]. Post-transcriptional regulation of LDLR is carried out through the modulation of its mRNA stability [29]. LDLR mRNA is a transcript containing a 2.5 kb-long stretch of 3′ untranslated region (3′UTR) [30] that has several cis-regulatory elements that control the rate of LDLR mRNA degradation by binding with trans-regulatory proteins [31], which can act as stabilizers or promoters of mRNA decay [32,33]. Finally, LDLR is also regulated post-translationally and this is mainly through PCSK9 which mediates its degradation, by binding to LDLR in the cell membrane and internalizing [34,35]. PCSK9 binding is highly specific for the EGF domain of LDLR because mutations in this domain interfere with PCSK9 binding and subsequently inhibit LDLR degradation [36,37]. Furthermore, LDL has been shown to compete with PCSK9 for the binding of LDLR, thus inhibiting its function [38]. It was also observed that the affinity of PCSK9 to LDLR substantially increases at acid endosomal pH relative to neutral cell surface pH, which is correlated with the post endocytosis process, where LDLR bound to PCSK9 is directed towards lysosomal degradation rather than recycling to the membrane again [38, 39].

The regulation of LDLR expression by non-sterol-dependent pathways has, so far, been poorly studied. However, it has been described, that certain cytokines (IL-1β, TNF-α and TGF-β) and the inflammatory environment could influence cholesterol metabolism and LDLR expression. This was observed mainly in hepatocytes, mesangial cells, and smooth muscle cells [40-42]. In breast cancer, Gallagher et al. showed that in mice with hyperlipidaemia conditions, triple-negative breast cancer cells with high LDLR expression are more proliferative while cells in which LDLR has been knocked down give rise to smaller tumours [43]. This study highlights the importance of LDLR expression on breast cancer progression. How LDLR expression is regulated in cancer cells is however poorly understood.

Contribution of the Immune microenvironment to breast cancer progression

The tumour microenvironment (TME) consists of the entire environment around tumour cells, this includes the surrounding blood vessels, immune cells, fibroblasts, signalling molecules and the extracellular matrix [44]. The interaction between tumour cells and their microenvironment is critical for tumour growth and progression. In response to the evolution of environmental conditions over time and oncogenic signals, TME changes continuously over the course of tumour progression, emphasizing the need to consider the influences of TME on cancer progression as a dynamic process [45]. One of the greatest pieces of evidence that tumorigenesis is affected by the deregulation of the microenvironment, is that in tissues with inflammation, generally there is a

higher incidence of cancer. This link was proposed in 1863 by Rudolf Virchow for the first time, as he observed that infiltrating leukocytes were a registered trademark of tumours [46]. Since then, several studies have contributed to the characterization of the TME [45].

Focusing on immune cells, these are extremely important to fight the tumour and control its growth and development. However, at some point, they alter their defence behaviour to favour tumour progression, doing the complete opposite [47]. Within tumour-promoting immune cells, we have tumour-associated macrophages (TAMs) that induce breast cancer cells to become more invasive through paracrine signalling [48] and mediate extravasation and metastatic growth in the lung [49]; myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Treg) involved in evasion and suppression of the immune system [50, 51]; and tumour infiltrating lymphocytes (TILs) that promotes angiogenesis [52, 53], induce epithelial to mesenchymal transition [54] and modulates the immune system [55, 56]; among others.

As it is clear a lot is known about how different immune cells may contribute with either pro or anti-tumoral signals. It is also evident that the system is complex and there is a big level of crosstalk between each cell type present at the tumours. The main players in such crosstalk are cytokines. Cytokines are small peptides responsible for autocrine, paracrine and endocrine signalling as immunomodulating agents. They include chemokines, interferons, interleukins, lymphokines, and tumour necrosis factors, but generally not hormones or growth factors. They act through cell surface receptors and are especially important in the immune system and in regulating the maturation, growth, and response capacity of certain cells. Cytokines are important in health specifically in host immune responses to infection, inflammation, and cancer [57]. Some of the most common cytokines present in the tumour are either pro-inflammatory like IFNγ, IL-1β, IL6, IL12 and TNFα [58] and an anti-inflammatory like TGFβ, IL4, IL10 and IL13 [59]. How cytokines present at the tumour microenvironment may affect cholesterol metabolism on cancer cells and what could be the consequences of this, is, however not known. Here we addressed the effect of some of the most prevalent cytokines at the tumour microenvironment as such, we tested the effect of tumour necrosis factor alpha (TNFα), growth factor beta transforming factor (TGFβ), interleukin-1β (IL-1β), interleukin-10 (IL10) and interferon-gamma (IFN-γ) on LDLR expression.

TNFα is a pro-inflammatory cytokine, most commonly, produced by immune cells that has several functions. Their role in tumour promotion was first suggested in mouse colon cancer. When TNF-α was inhibited, they were less susceptible to colon cancer [60]. There is more and more evidence that TNFα is involved in the activation of the NF-κB pathway, which can increase the survival of tumour cells [61], and in the activation of the PKCα and AP1 pathway, which regulates both cell death and growth [62]. On the other hand, studies have shown that TNFα, produced by cytotoxic T cells and macrophages, can have the opposite effect helping to fight the disease. Therefore, the role of TNFα in tumour progression and its general effects is still controversial [63].

TGFβ is known, depending on the context, as a pro-inflammatory cytokine, leading to the generation of Th17 cells that have pro-inflammatory activity [64], and an anti-inflammatory cytokine, inhibiting cell growth by activating the SMAD4 signalling pathway, being a possible defence against the tumour. On the opposite, it has also been proven that TGFβ promotes the process of epithelial-mesenchymal transition (EMT), a necessary step to form metastases [65].

IL-1β is a pro-inflammatory cytokine, produced mainly by macrophages, which is extremely important for a competent immune response [66]. It has functions such as activating the NF-κB

pathway leading to promoting the expression of defensins and cathelicidin [67]. It has been demonstrated in melanoma cells, that IL-1β will promote angiogenesis and induce the infiltration of neutrophils and monocytes, consequently, being able to create a selective pressure to select the most suitable cells [68].

IL10 is an important regulatory cytokine with an anti-inflammatory effect [69]. IL10 is produced mainly by monocytes and to a lesser extent by type 2 helper T lymphocytes (Th2), mast cells, regulatory T cells and B cells [70]. Presents polymorphisms (SNPs) and several groups report associations between polymorphism and cancer risk [71]. This cytokine has the ability to inhibit the activation and effector function of T cells, monocytes and macrophages [72]. However, the role of IL-10 in breast cancer is controversial. Interleukin 10 has both pro and antitumour effects [73]. It has been reported that serum from breast cancer patients IL10 concentrations are higher than in healthy individuals [74] and that this was associated with poor clinical outcomes [75]. IL10 also promotes tumour cell proliferation and metastasis [76] and inhibits T cell proliferation and function [77]. It also prevents APCs from obtaining tumour antigens [78]. Finally, IL10 also negatively affects the induction of angiogenesis [79].

IFNγ is produced mainly by natural killer (NK) and natural killer T (NKT) cells in innate immunity while, during the adaptive immune response, CD8+ and CD4+ T-cells are the major sources of IFNγ [80]. IFNγ is a proinflammatory cytokine that has essential roles in intercellular communication during immune responses and in the defense of the host against viral and bacterial infections, as well as against cancer [81]. These roles of IFNγ inspired the scientific community to try to use this cytokine as a clinical therapy for a variety of diseases such as cancer. However, the results of these cancer-related clinical trials were not very consistent [82] which led to the raising of some pertinent questions, for example, does IFN-γ contribute to tumour regression or stimulate tumour growth? This and many other questions still need to be answered. Pieces of evidence are emerging that IFN-γ may also be involved in the opposite process of promoting the tumour, more specifically in the tumour equilibrium and evasion stages, involving proliferative and anti-apoptotic signals and escape from recognition and cytolysis by cytotoxic T lymphocytes and NK cells [83]. Survival of circulating tumour cells and increased metastatic potential have been reported to be induced by low doses of IFNγ, either generated at the tumour site by infiltrating cells or during cytokine therapy [84, 85]. Furthermore, IFN-γ have been suggested to also contribute to the formation of metastases through the transformation of cancer stem cells into metastatic cancer stem cells [86]. IFNγ has also been shown to have a metastatic role in triple-negative breast cancer, where the loss of the tumour suppressive transcription factor Elf5, along with its ubiquitin ligase FBXW7, activated IFN-γ signalling and promoted tumour progression and metastasis, through the stabilization of IFNGR1. Furthermore, this signalling increased PD-L1 expression and led to immune suppression [87]. It has also been shown that IFNγ impairs the T cell immune response by inducing PD-L1 expression in lymphatic endothelial cells, which limits the migration of cytotoxic T cells to the TME [88]. In addition, IFN- γ can also induce tumour-specific T cell apoptosis [89]. Finally, it was also observed that in breast adenocarcinoma, chronic exposure to low levels of IFNγ, caused tumour development and induction of PD-L1, PD-L2, CTLA-4 and Foxp3 molecules that controlled, at least partially, the tumour immune evasion [90]. Overall, IFNγ affects tumour growth not only directly, but also through the modulation of the immune response at the TME. Regarding cholesterol metabolism, interferons have been shown to decrease LDL-cholesterol concentrations (20-50%) and apo B levels through a reduction in LDL and apo B rate production [91]. More specifically speaking of IFNy, this molecule is able to reduce the efflux of cholesterol in endothelial cells, monocytes and macrophages, through the

inhibition of cholesterol hydroxylase [92, 93]. As for LDLR, the relationship between IFNγ and this receptor is mainly unknown.

Project's aim and its impact on society

It is becoming increasingly evident that cholesterol metabolism affects breast cancer progression and that an aggressive behaviour is promoted by LDL. The LDLR is the main receptor for LDL and others have shown that high LDLR leads to more proliferative breast cancer cells and bigger tumours in mouse models. How the LDLR expression and LDL uptake in general is regulated at the tumour is however not known. Here we proposed to study cholesterol dependent and independent regulation of LDLR in breast cancer cells. Considering the cholesterol-independent mechanism, we hypothesize that there are factors at the TME that increase LDLR expression, which leads to increased cholesterol uptake by cancer cells and originate a more aggressive behaviour. As cytokines are an important component of the TME and have been previously shown to regulate LDLR expression in other cellular contexts, here we investigated whether some of these cytokines could induce LDLR expression and it´s functional consequences on breast cancer cells. As such we set up the following objectives: 1- To characterize the regulation of the LDLR-receptor by sterol-dependent mechanisms. 2- To test a small panel of cytokines known to be present at the tumour microenvironment for its ability to induce LDLR expression in breast cancer cells. 3- Having identified a cytokine that induces LDLR expression in BC cells further characterize the mechanism by which it occurs. 4- Having identified a cytokine that induces LDLR expression in BC cells further characterize it´s consequence in LDL uptake and in the LDL-induced cellular aggressive behaviour. We believe this knowledge will contribute to better understand how cholesterol metabolism and the crosstalk between cholesterol metabolism on cancer cells and cytokine production at the tumour microenvironment may affect cancer progression. This will allow the better understanding of the complex set of molecular and cellular interactions that occur in cancer which we believe will contribute to better prevent, predict and treat breast cancer in the future.

Materials and methods

Cell lines and incubation conditions

Three breast cancer cell lines were used in this project. Two human breast cancer cell lines, MDA-231 (ATCC, USA, Virginia) and MCF-7 (ATCC, USA, Virginia), and one mouse breast cancer cell line, 4T1 (ATCC, USA, Virginia).

Both cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific) and 1% penicillin-streptomycin (Thermo Fisher Scientific) at 37°C with 5% CO2. Cells were passaged routinely to maintain 50-80 % confluency.

For passaging, cells were first washed with PBS and then suspended using 0.25% trypsin (Thermo Fisher Scientific, USA, Massachusetts) until cells detached, and then trypsin was inactivated with DMEM + FBS + penicillin-streptomycin.

For plating, cells were treated in the same way of passaging and after trypsinization cells were counted using a haemocytometer and resuspended in the appropriate volume of media in order to seed 2x10^5 cells in 6-well plates for LDLR protein and gene expression experiment and in 24-well plates for migration assay. 1x10^5 cells in 12-well plates were used for cholesterol quantification experiments and 0.5x10^5 in 24-well plates for proliferation assay.

For cholesterol enrichment and depletion, with Methyl B Cyclodextrin: cholesterol complexes and LDL, the day after plating, the culture medium was changed to an FBS-free DMEM supplemented with 1% penicillin-streptomycin. One hour later of medium change, LDL (Calbiochem) was added in a final concentration of 0.1 mg/ml to its respective well, except for the negative and positive control, added in a final concentration of 0.2 mM. The cells were then incubated for 6 hours at stable physiological conditions. In experiment two, LDL was added to its respective well 22 hours after the medium change and IFNγ addition. The cells were then incubated for 2 hours at stable physiological conditions.

Total cholesterol quantification with Amplex® Red Cholesterol assay

The Amplex® Red Cholesterol Assay Kit gives us a simple fluorometric method for quantifying cholesterol through fluorescence reading. The assay is based on a reaction that detects both free cholesterol and cholesteryl esters. Cholesteryl esters are hydrolyzed by cholesterol esterase to cholesterol, which is further oxidized by cholesterol oxidase to produce H_2O_2. H_2O_2 is then detected using Amplex® Red reagent which in the presence of horseradish peroxidase (HRP), produces highly fluorescent resorufin.

For cholesterol extraction, cells were first placed on ice, washed with ice-cold PBS 2 times, and then lysed with Amplex® Red reaction buffer 1x (Invitrogen). Lysates were centrifuged at 12000 rpm for 20 minutes at 4°C. The supernatants were extracted and stored at -20°C until they were needed for subsequent steps.

Wells from a 96-well plate was used with 5 µl of each sample, or with cholesterol reference standard in known concentrations diluted in reaction buffer. A working solution containing 2

U/mL HRP, 2 U/mL cholesterol oxidase, and 0.2 U/mL cholesterol esterase was added to each well. 50µL of sample and 50µL of the working solution was used to start the reaction. Next, the reactions were incubated for at least 30 minutes at 37°C, protected from the light. The fluorescence intensity was analyzed at an excitation wavelength in the range of 530–560 nm and emission detection at 590 nm using a Tecan Plate Reader (model Infinite M200, Tecan, Switzerland, Männedorf). To make calibration curves, the relation between cholesterol reference standard concentrations and fluorescence intensities were used, which enabled the quantification of the extracted cholesterol.

Incubation with cytokines and LDL

The day after plating, the medium was changed to a fetal bovine serum-free DMEM supplemented with 1% penicillin-streptomycin, and each cytokine was added to its respective well, except for the untreated and LDL conditions, that remained cytokine-free. TGF-β (Peprotech, USA, New Jersey), IL-1β (Peprotech), IFN-γ (Peprotech) and IL10 (Peprotech) were added in a final concentration of 5 ng/ml in the wells, and for TNF-α (Peprotech), was added in a final concentration of 50 ng/ml. LDL was used in a final concentration of 0.1 mg/mL. The cells were then incubated for 24 hours at stable physiological conditions.

Protein Extraction

For protein extraction, cells were first placed on ice, washed with ice-cold PBS 2 times, and then lysed with RIPA buffer (Merck) that was prepared with cOmplete™ Protease Inhibitor Cocktail (Roche, Switzerland, Basel) or when necessary with phosphatase inhibitors. Lysates were then moved to a cold eppendorf on ice and centrifuged at 12000 rpm for 20 minutes at 4 °C. The supernatants were extracted to new mini-eppendorfs to which a solution of Laemmli (Bio-Rad) with 2% dithiothreitol (Bio-Rad) was added in a proportion of 1 portion of Laemli to 4 portions of lysate. The resulting solutions were heated at 95°C for 5 minutes and stored at -20°C until they were needed for subsequent steps.

Total protein quantification with Lowry protein assay

The total protein quantity was determined using the DC Protein Assay (Biorad) which is based on the Lowry protein assay. Wells from a 96-well plate was used with a portion of each sample, or with bovine serum albumin (BSA) (Merck) in known concentrations diluted in RIPA lysis buffer. Both A' (made from A and S solutions in a proportion of 98% and 2%, respectively) and B solutions (Bio-Rad) were added to each well in a concentration of five times and forty times the amount of sample or BSA, respectively. After 15 minutes the absorbances were analyzed at a wavelength of 750 nm using a Tecan Plate Reader. To make calibration curves, the relation between BSA concentrations and absorbances were used, which enabled the quantification of the extracted protein.

Western Blot assay

The total extracted proteins were separated using 10% SDS-PAGE gels, which were made using acrylamide (Bio-Rad), Tris hydrochloride buffers (Tris-HCl) (Bio-Rad), sodium dodecyl sulfate (SDS) (Thermo Fisher Scientific), ammonium persulfate (APS) (Merck) and tetramethylethylenediamine (TEMED) (NZYTech, Portugal, Lisbon). A Precision Plus Protein™ Dual Color Standards ladder (Bio-Rad) was used to access the proteins' molecular weight. The electrophoreses were followed by protein transference to nitrocellulose membranes at 40V for 1h and 30min in 20% methanol (Avantor, USA, Pennsylvania), 10% Tris/L glycine buffer (Bio-Rad). The membranes were blocked afterwards using a 5% milk solution made from powdered milk (Nestlé, Switzerland, 7 Vevey) in TBST (10% Tris-buffered saline 10x/0,01% Tween-20) for one hour at room temperature followed by a rinse with TBST. The membranes were then incubated with the primary antibodies: goat anti-human LDLR (Novus Biologicals, USA, Colorado), mouse anti-human PCSK9 (ThermoFisher, USA, Massachusetts), rabbit anti-human STAT1, rabbit anti-human pSTAT1, rabbit anti-human AKT, rabbit anti-human pAKT, rabbit anti-human S6, mouse anti-human pS6 (1:1000 in TBST) and mouse anti-human β-Actin (Merck) (1:5000 in TBST) O/N at 4°C followed by three 10 minutes washes with TBST. Upon this, membranes were incubated with the secondary antibodies: donkey anti-goat horseradish peroxidase-labelled (Santa Cruz Biotechnology, USA, Texas) and Goat anti-mouse horseradish peroxidase-labelled (Promega, USA, Wisconsin), respectively, (1:5000 in 5% milk solution), for 1 hour at room temperature, followed by three 10 minutes washes with TBST. Finally, enhanced chemiluminescence (ECL) detection reagents (GE Healthcare, USA, Chicago) were mixed and the membranes were immersed in them for one minute before the chemiluminescence was detected using an Amersham Imager 680 (GE Healthcare). To analyze the Western Blot results, band intensities were obtained using the image analysis program ImageJ, which provided the absolute intensity. The absolute intensities were then normalized using β-actin as a baseline for protein production and, afterwards, each sample's normalized intensity was normalized again using the control intensity in order to represent the relative intensity and to better observe up- and downregulations compared to the control sample.

Immunofluorescence

Frozen mouse tumour (4T1) slices were used to visualize LDLR expression throughout the tumour and also to see if there was any co-localization between immune cells and LDLR expression of tumour cells. For this, firstly. a perimeter around the tumour sections was drawn with a PAP pen and then the sections were fixed using paraformaldehyde (Merck) (4% in PBS) for 20 minutes, followed by one wash in PBS. The sections were then subjected to a blocking process using BSA (Merck) to reduce the background signal in fluorescence microscopy. Next, samples were incubated with the primary antibodies: goat anti-mouse LDLR, rat anti-mouse CD11b, and rat anti-mouse NKp44+. For this, samples were immersed in a small drop of a 1:100 solution of primary antibody in PBS, stored O/N at 4°C in a humid chamber. The slides were then washed 3 times with PBS and for the second incubation, a mix was made using the secondary fluorophore labelled antibody anti-goat and anti-rat which emitted light at a wavelength of 633nm and 488nm, respectively, in a concentration of 1:500 (Alexa, Thermo Fisher Scientific) in PBS. The Slides were incubated in small drops of the mix for 1 hour at room temperature and then covered with a drop of VECTASHIELD® Antifade Mounting Medium with 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, USA, California) and finally with a coverslip. The

coverslip was subsequently fixed to the slide with nail polish. The microscope slides were visualized in a Zeiss Cell Observer fluorescence microscope.

RNA extraction and cDNA preparation

The extraction of RNA was performed on MDA-231 cell samples with NZY Total RNA Isolation kit, following the manufacturer's instructions. The RNA was quantified by spectrophotometry in NanoDrop (ThermoFisher), using its analysis software. The quality of the samples was evaluated by the A260 / A280 ratio. The cDNA was prepared using the NZYTech kit, according to the manufacturer's instructions. The obtained cDNA was stored at -20 ° C until later analysis.

Real-Time PCR

Transcripts LDLR and PCSK9 were selected. For these, primers (Forward-GATACCAAGGGCGTGAAGAG, Reverse-AAGCCATGAACAGGATCCAC) and (Forward-CATGTCTTCCATGGCCTTCT, Reverse-GTAGTCGACATGGGGCAACT) were used, respectively. RT-qPCR was performed on a MicroAmp Optical 384-Well Reaction Plate (Applied Biosystems), using a real-time PCR ViiA 7. Two technical repeats were performed. The reaction mix was composed of 5uL of NZYSpeedy qPCR Green Master Mix, 0.8 µl of forward primer (10 µM), 0.8 uL of reverse primer (10 µM), diluted cDNA and water to make the final volume of 10 µl. cDNA concentration in the mix was 800 ng mL-1. The running consisted of 1 cycle at 95°C for 2 min and 40 cycles at 95 °C for 5s, followed by 15s at 65 °C. The values were exported to Excel and the quantification of the relative gene expression was done through the ΔΔCt method (Livak and Schmittgen 2001). RNase P was used as a reference gene. It was considered that a fold expression higher than 1 represented an upregulation of the considered gene.

Proliferation Assay

MDA-231 cells were seeded at a density of 0.5 x 10^5 cells/mL in round bottom 24-well plates in 500uL DMEM + FBS 10%. After 24h of incubation, the medium was replaced by DMEM only and the conditions were added (LDL, IFNγ and LDL+IFNγ). The number of viable and not viable cells were counted with hemocytometer at 0h, 24h, 48h and 72h using the trypan blue exclusion assay.

Wound Healing Assay

MDA-231 cells were seeded at a density of 2 x 10^5 cells/mL in round bottoms in 500uL DMEM + FBS 10%. After 24h of incubation, the medium was replaced by DMEM only for 24h. Two hundred microliters tips were used to make a scratch in the centre of the well and after washing with PBS, the medium was replaced by DMEM supplemented with Mitomycin C (Merck) at a final concentration of 0.5uM to block cell proliferation and with the conditions (LDL, IFNγ and LDL+IFNγ). Cells were observed under 4x objective on Zeiss Primovert microscope coupled with the Zeiss AxioCam. The pictures were taken at 0h, 12h, 24h and 36h.

Results

LDLR regulation by cholesterol in BC cells

In order to study the sterol-dependent regulation of LDLR expression in breast cancer cells, MDA-MB-231 cells and MCF7 cells were exposed to either Mehyl-Beta-Cyclodextrin + cholesterol complexes or to LDL. As both treatments were able to increase intracellular cholesterol levels when compared to control (Figure 1) we then adressed the expression levels of LDLR upon LDL treatment by western blotting.

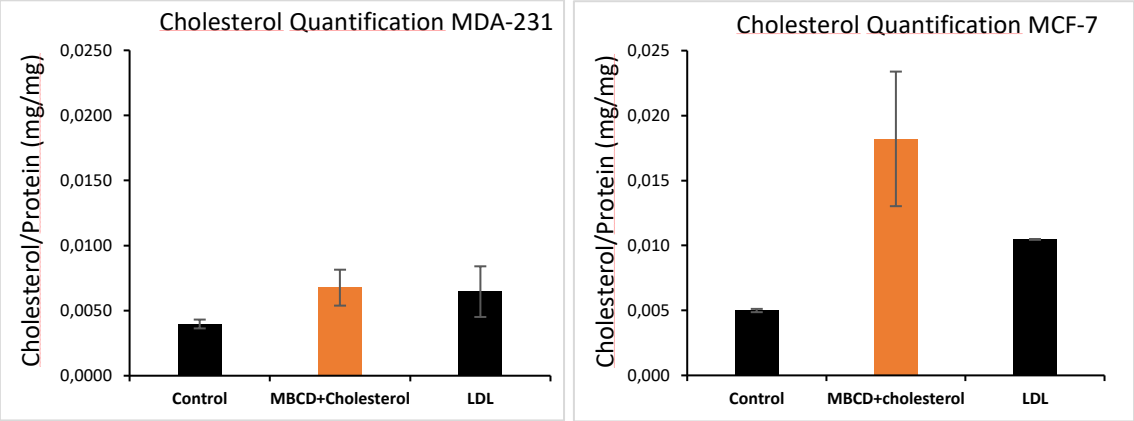


Figure 1– Intracellular cholesterol modulation by exogenous cholesterol in breast cancer cells. Cholesterol quantification in MDA-231 cells (left, n=3) and MCF-7 cells (right, n=2) upon treatment with Methyl Beta Cyclodextrin:cholesterol (MBCD+cholesterol) complexes and LDL. Each bar represents the mean ± SD of cholesterol/protein values.

As such, cells were exposed to LDL overtime and a western blot for the LDLR was performed, as well as cholesterol quantification at each time point (Figure 2A, 2B). The immunoblots revealed a band with a molecular weight (MW) of a bit over 150 kD when incubated with the anti-LDLR antibody (Figure 2C). Overall, the results obtained suggest that, in MDA-231, LDLR is downregulated by LDL. These results suggest that a normal negative feedback loop is taking place in this cell line upon exposure to LDL. When treated with LDL, cells start to internalize cholesterol and consuming it. As time passes, the increase in cholesterol in the cells will induce, through the SREBP, the inhibition of the LDLR gene expression, decreasing the amount of LDL receptors in the membrane, which in turn decreases the internalization of cholesterol.

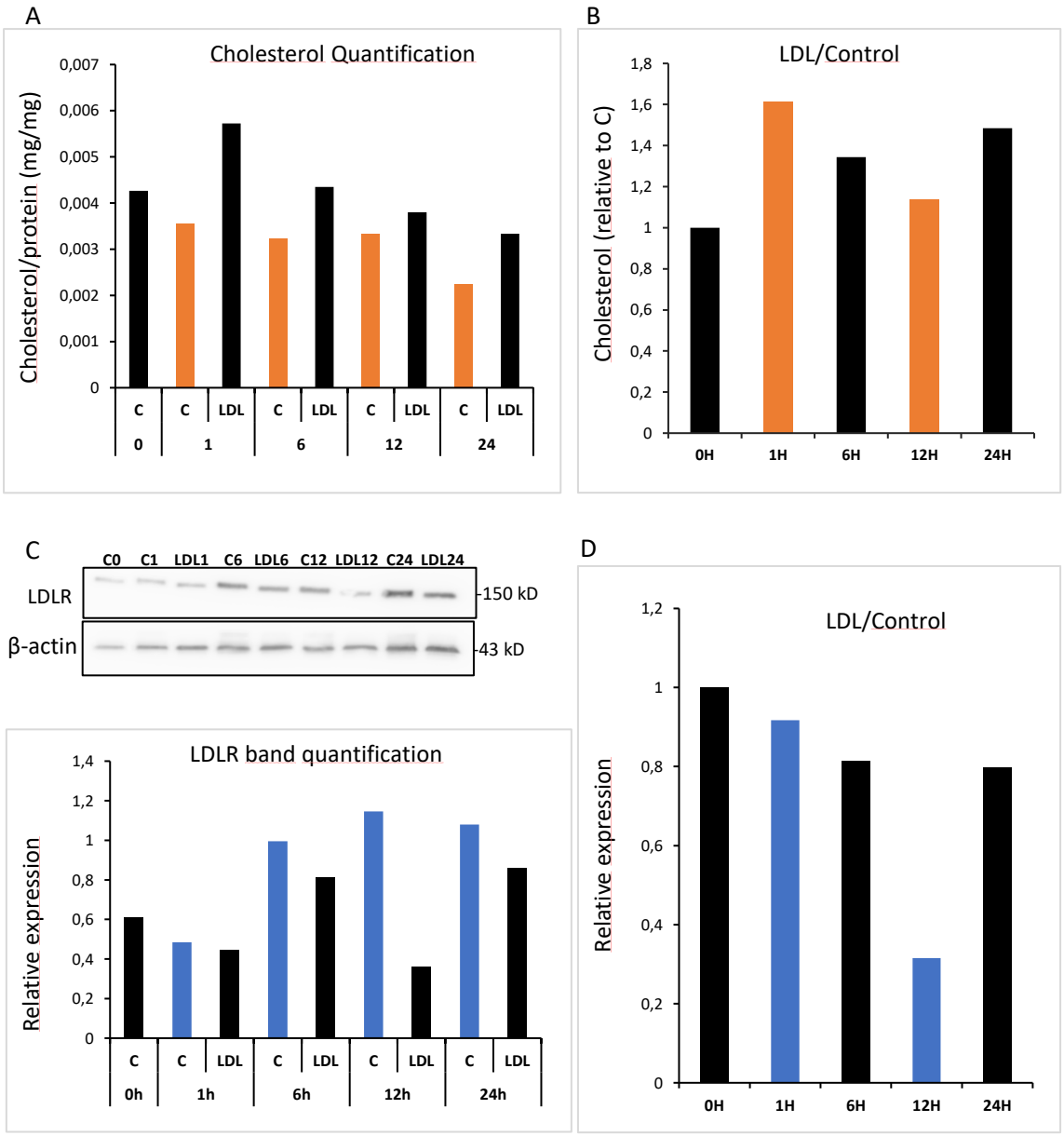


Figure 2 – A) Cholesterol quantification in breast cancer cells MDA-231 treated with or without LDL for 1 hour, 6 hours, 12 hours and 24 hours. B) Relative cholesterol values (which are calculated by the ratio between LDL and control) in comparison with the control 0 hours. C) Immunoblot bands and their respective LDLR band quantification for MDA-231 cell line, treated with LDL and lysed at 1 hour, 6 hours, 12 hours and 24 hours. D) Relative LDLR expression values (which are calculated by the ratio between LDL and control) in comparison with the control 0 hours. The upper band in the immunoblot corresponds to LDLR while the lower band corresponds to β-actin. The values determined in the band intensity quantification correspond to the ratio between the intensities of the LDLR band and the β-actin band. n=1.

LDLR regulation by cytokines

After having confirmed that we are able to detect variations of the LDLR expression upon stimuli in both the MDA-231 and the MCF7 cell lines we then tested our main hypothesis. That TME cytokines modulate the expression of the LDLR. For that we tested a small panel of cytokines: IFNγ, IL1β, TNFα, TGFβ and IL10 and western blot to analyse the LDLR expression. At this step we also included a third breast cancer cell line, the mouse 4T1 cell line (Figure 3C).

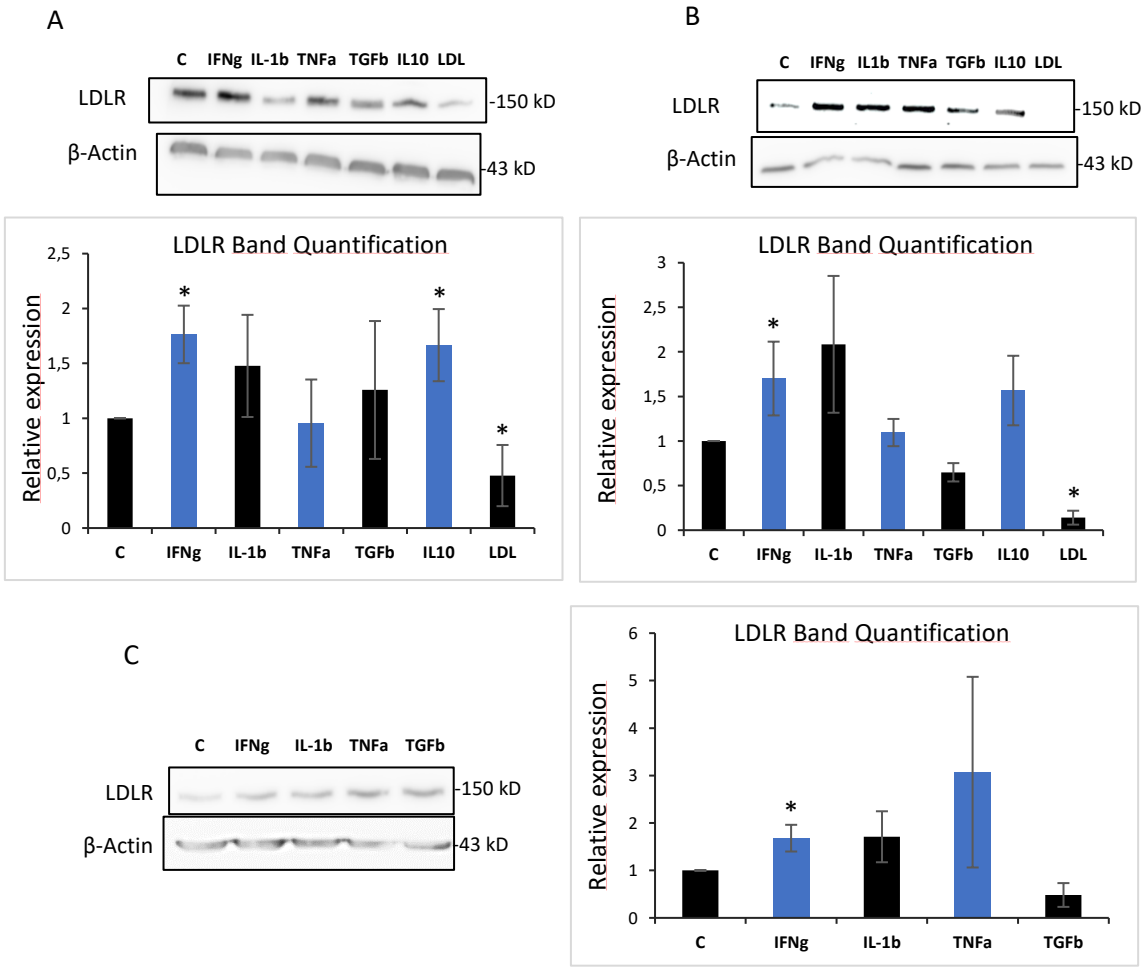


Figure 3 – Immunoblot bands and their respective relative LDLR band quantification for both MDA-231 (A), MCF-7 (B) and 4T1 (C) cell lines, treated with various cytokines. The upper band in the immunoblot corresponds to LDLR while the lower band corresponds to β-actin. The values determined in the band intensity quantification correspond to the relative LDLR band intensity values (which are calculated by the ratio between the intensities of the LDLR band and the β-actin band) in comparison with the control band's relative intensity. n=3, * p value <0.05.

Before analysing the effect of each cytokine on LDLR expression we tested whether any of these cytokines at the concentrations used were either toxic or dramatically affected cell division on these cell lines. For this, we used a sulforhodamine B assay, that allows the measurement of protein mass upon different stimuli. We could not detect any major effect of any of the cytokines used in protein mass which suggested that any of these cytokines was killing or dramatically inhibiting cell proliferation (Supplementary figure 1). We also checked whether any of these cytokines would promote cholesterol synthesis, by measuring intracellular cholesterol levels upon 24 hours treatment with the cytokine panel in the absence of serum and we did not observe any increase in cholesterol content, suggesting, that none of the cytokines induces cholesterol

synthesis (Supplementary figure 2). As for LDLR expression, the result of three independent experiments using the three different cell lines showed a statistically significant upregulation of LDLR in MDA-231, MCF-7 and 4T1 cells treated with IFNγ. IL10 treatment also led to increased expression of LDLR in cell lines in the MDA-231 and MCF-7. TGFβ and TNFα, in turn, presented highly variable values both between lines and within the cell line itself.

IFNγ induces cholesterol uptake by BC cells

Having identified IFNγ as an inducer of LDLR expression we then tested whether this would have functional consequences. For this we chose the MDA-231 cell line, as the majority of data from our lab on the effect of LDL on breast cancer aggressive behaviour was obtained using this cell line. The first consequence of IFNγ-induced LDLR expression we tested was whether IFNγ leads to increased LDL uptake. For that, we used different concentrations of IFNγ and exposed cells to IFNγ for 22 hours. After this, we added LDL for two hours and finally collected cells for cholesterol quantification. (Figure 4).

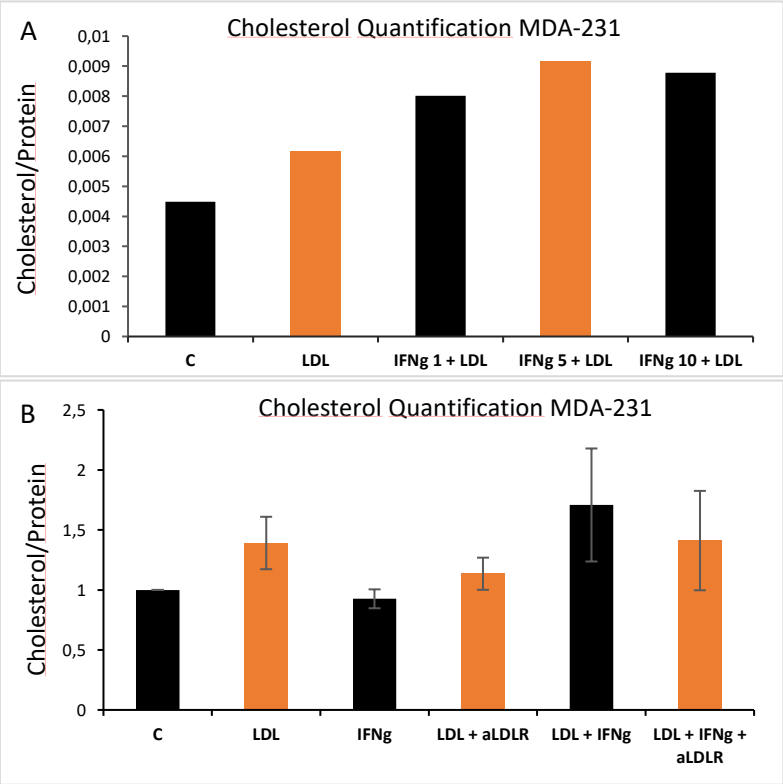


Figure 4 – Amplex Red Cholesterol Quantification Assay for MDA-231. A) Cells were treated with LDL only (positive control) and with LDL paired with IFN-γ in different concentrations (1, 5 and 10 ng/mL). In the negative control (C) neither IFN-γ or LDL was added. n=1. B) Cells were treated with LDL only, IFN-γ only (5ng/mL), LDL paired with IFN-γ, LDL paired with αLDLR and LDL paired with IFN-γ and αLDLR. n=5. IFN-γ was added for 24h, αLDLR for 2h:30min and LDL for 2h.

An increase in the total cholesterol was observed in every condition when compared to the control. Also, for every condition treated with LDL and IFNγ, independently of the cytokine concentration used, there was an increase of the total cholesterol when compared to the condition with LDL only (Figure 4A). The IFNγ concentration that presented the highest cholesterol content was 5 ng/mL, which was the concentration used from now on. Next, we wanted to see if the higher cholesterol uptake was, in fact, due to the increase of LDLR expression through IFNγ. For this, we use an LDLR blocker, αLDLR (Figure 4B). We can observe that the blocker is working, as it reversed the effect when added with LDL. Cells treated only with IFNγ are shown again to not affect intracellular cholesterol levels. Overall the results suggest that the increased expression of LDLR through IFNγ leads to increased LDL internalization and cholesterol accumulation on cells.

Knowing this we then tested whether this increased LDL uptake would lead to a more aggressive behaviour by tumour cells. For that we performed proliferation and migration assays. As for proliferation MDA-231 cells numbers were measured after 24 hours and 48 hours. As shown in Figure 5, cells were seeded (0.5 x 10^5 cells/well) under 4 different conditions (C, LDL, IFNγ and IFNγ + LDL) and cell counts were performed 24 and 48 hours after seeding.

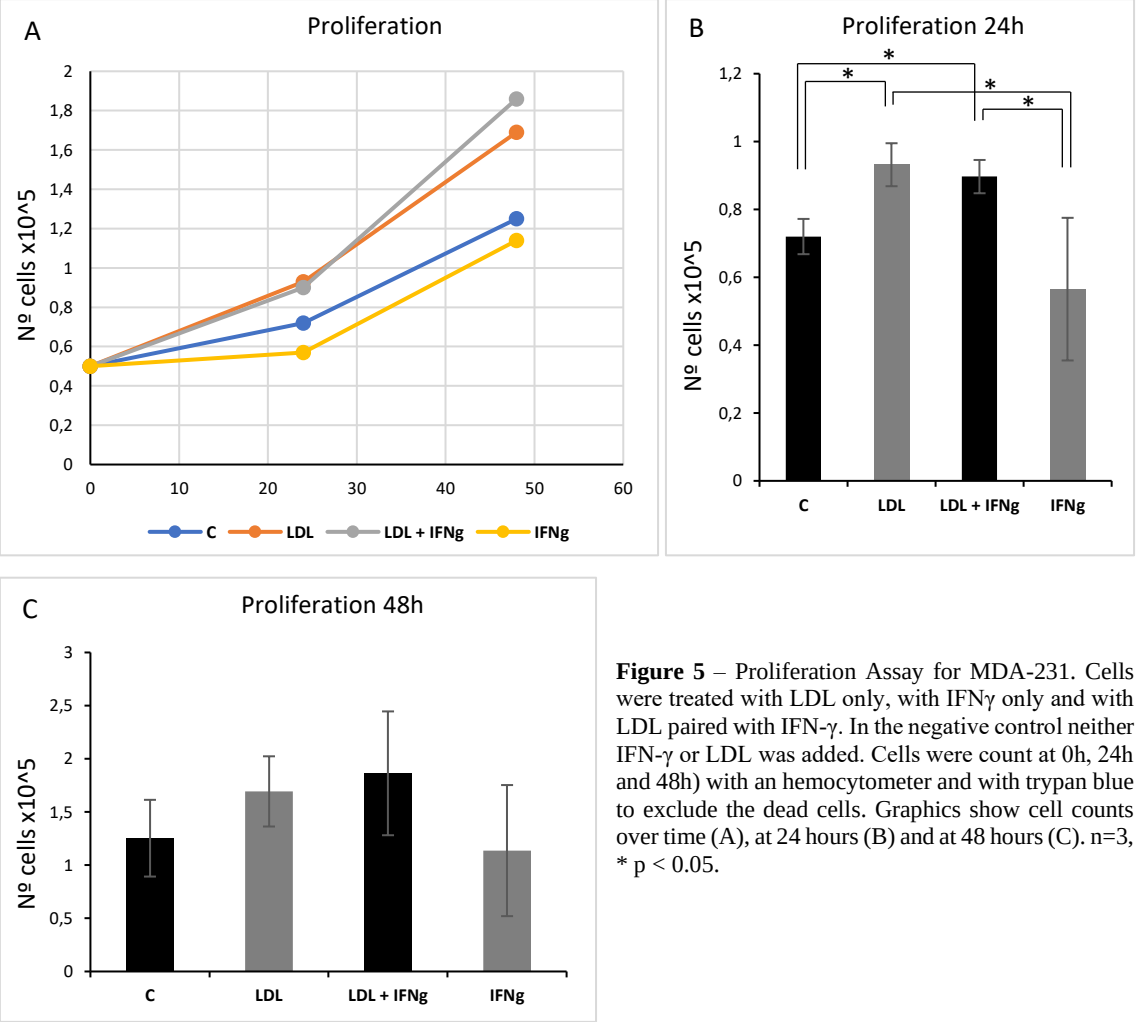


Figure 5 – Proliferation Assay for MDA-231. Cells were treated with LDL only, with IFNγ only and with LDL paired with IFN-γ. In the negative control neither IFN-γ or LDL was added. Cells were count at 0h, 24h and 48h) with an hemocytometer and with trypan blue to exclude the dead cells. Graphics show cell counts over time (A), at 24 hours (B) and at 48 hours (C). n=3, * $p < 0.05$.

As expected, LDL increased cell proliferation in breast cancer cells, while IFNγ decreased proliferation compared to control. However, it was observed that the highest increase in cell proliferation, after 48h, was in cells treated with IFNγ + LDL, however, it's not statistically significant. This result may indicate that the increased uptake of LDL-cholesterol, due to the action of IFNγ on the LDLR, is also increasing the rate of proliferation of breast cancer cells.

In the migration assay, we used the wound healing assay, which examines cell migration in response to the mechanical scratch. Images of scratch areas from the time point 0h and 24h are illustrated in Figure 6.

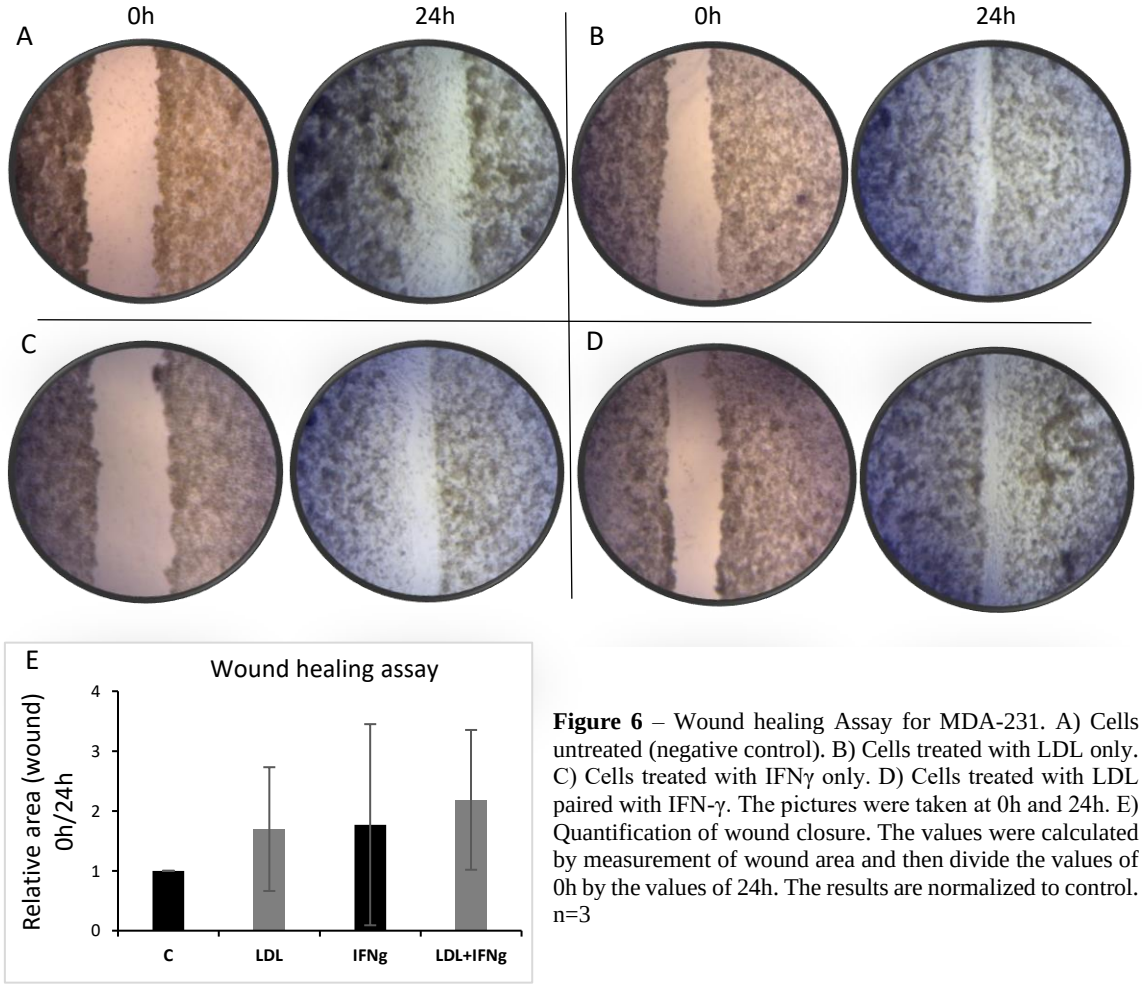


Figure 6 – Wound healing Assay for MDA-231. A) Cells untreated (negative control). B) Cells treated with LDL only. C) Cells treated with IFNγ only. D) Cells treated with LDL paired with IFN-γ. The pictures were taken at 0h and 24h. E) Quantification of wound closure. The values were calculated by measurement of wound area and then divide the values of 0h by the values of 24h. The results are normalized to control. n=3

Even without showing statistically significant results, our data suggest that treatment with LDL (Figure 6B) and IFNγ (Figure 6C) caused an induction of cell migration relative to the control (Figure 6A). In the condition where LDL + IFNγ was added (Figure 6D) there was a tendency for an even further increased closing of the wound. The rate of wound closure was quantified and is represented in a histogram in Figure 6E. These results suggest that, in addition to the effect of LDL in promoting the migratory behavior of breast cancer cells as expected, the effect of IFNγ together with LDL further enhances this behavior. However, even though it is not possible to say whether this is due to the fact that IFNγ leads to more uptake of LDL through the LDLR, because IFNγ alone induces more cell migration. In sum, there seems to be a trend towards increased migration, but the experiment would have to be optimized and the "n" increased, since LDL, which should give an increase in migration (positive control), does not give a statistically significant increase.

Having identified IFNγ as a regulator of LDLR expression we then explored the molecular mechanisms by which this occurs. As a first question, we addressed whether this is happening due to an effect at the level of gene expression or the level of post-transcriptionally level. To know if IFNγ was acting at the level of the *LDLR* gene, a real-time RT-PCR analysis was performed on MDA-231 cells (Figure 7A). For this, 2 genes were selected to study, *LDLR* and *PCSK9*. Cells were treated with IFNγ or LDL for 24h.

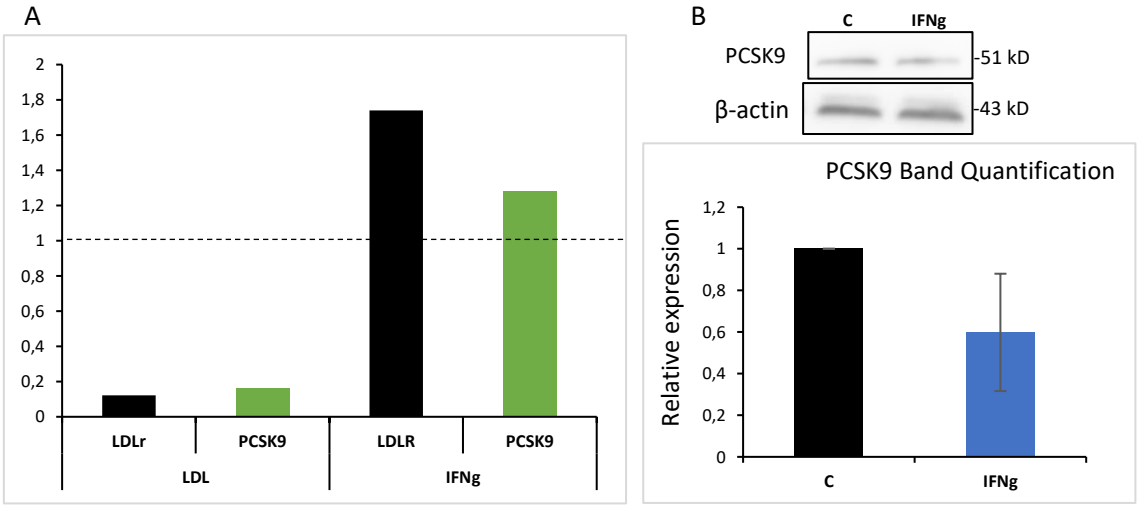


Figure 7 – A) LDLR and PCSK9 gene expression in MDA-231 cells treated with IFNγ or LDL when compared to control, for 24 hours. The values represented in the graph were obtained by RT-qPCR. Values were normalized to the Ct values of a reference gene, RNAseP. An increase in expression compared to the control is only considered when the value is higher than 1, represented by a dashed line. n=1. B) Immunoblot bands and their respective relative PCSK9 band quantification for MDA-231 cell line, treated with IFNγ. The upper band in the immunoblot corresponds to PCSK9 while the lower band corresponds to β-actin. The values determined in the band intensity quantification correspond to the relative PCSK9 band intensity values (which are calculated by the ratio between the intensities of the PCSK9 band and the β-actin band) in comparison with the control band's relative intensity. n=3.

From the results, we can verify that the cells treated with LDL had a considerable decrease in *LDLR* and *PCSK9* expression, as expected and as such serves as a positive control of the experiment. As for the treatment with IFNγ, we can observe that after 24h there is an increase in the expression of *LDLR*. As for *PCSK9*, the data suggest that IFNγ treatment also induces its expression. In summary, this data suggest that IFNγ is inducing LDLR expression at the level of transcription, however, care needs to be taken to draw major conclusions, as the experiment should be repeated.

At the same time, we also analyzed the expression of PCSK9 upon IFNγ treatment at the protein level by western blot (Figure 7B). It was previously verified that in these breast cancer cells, PCSK9 would also be involved in the degradation of LDLR. For this, a PCSK9 blocker, α-PCSK9, was used and LDLR protein levels were then measured. We observed that with the blocking of PCSK9 there was a substantial increase in LDLR as expected (Supplementary figure 3). The immunoblots revealed a band with a molecular weight of a bit over 55 kD when incubated with the anti-PCSK9 antibody. The results showed a tendency for a decrease of PCSK9 upon IFNγ treatment, however, a big variation in the expression of PCSK9 throughout the repetitions

was observed and no statistically significant differences could be detected between experimental conditions. Taken together, our data suggest that IFNγ regulate LDLR expression at the level of transcription and not through downregulation of PCSK9, however, repetition of these experiments are required.

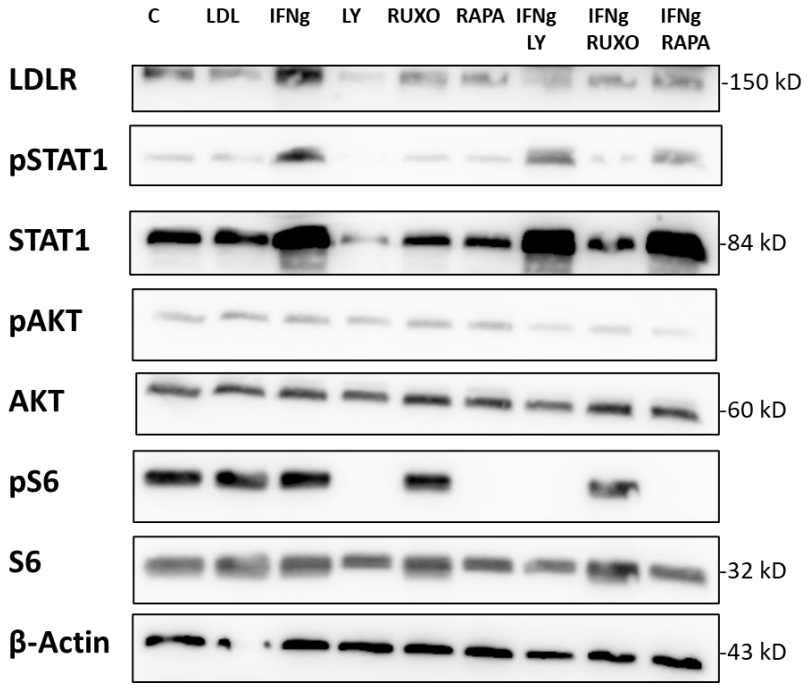


Figure 8 – Immunoblot bands of LDLR, pSTAT1, STAT1, pAKT, AKT, pS6 and S6 for MDA-231 cell line, treated with IFNγ, LY, Ruxolitinib, Rapamycin and LDL for 24h. β-Actin were used as loading control. n=1.

Finally, we analyzed which downstream signalling pathways were being activated upon IFNγ treatment and were related to IFNγ-induced increased LDLR expression. IFNγ is known to act mainly via the JAK/STAT1 signalling pathway, however less canonical signalling pathways downstream of IFNγ have been described. These include the activation of PI3K, AKT and mTOR. In order to identify which of such pathways was acting downstream of IFNγ in our system, a Western Blot assay was carried out using specific antibodies for phosphorylated STAT1, AKT and S6. Additionally, we also used different inhibitors for each of the pathways. Ruxolitinib an inhibitor of the JAK2, LY294002 an inhibitor of PI3K and Rapamycin an inhibitor of mTOR activity, to see if inhibiting any of these pathways would revert the IFNγ-induced LDLR expression (figure 8). With the results obtained, we can see that IFNγ is signalling through the JAK/STAT1 pathway as the cells treated with IFNγ had much more phosphorylated STAT1 than the control cells, and when this pathway was inhibited with Ruxolitinib, there was a reversion of the effect. As for the other pathways, our results do not demonstrate that IFNγ is inducing their activation, however, the use of the inhibitors suggest that all are associated with the regulation of LDLR expression as even without IFNγ stimuli, they lead to decreased LDLR expression.

LDLR expression *in vivo*

As our last experiment, we characterized the LDLR expression *in vivo* in breast cancer tumour xenografts of the 4T1 cell line in immunocompetent mice with and without a high cholesterol diet.

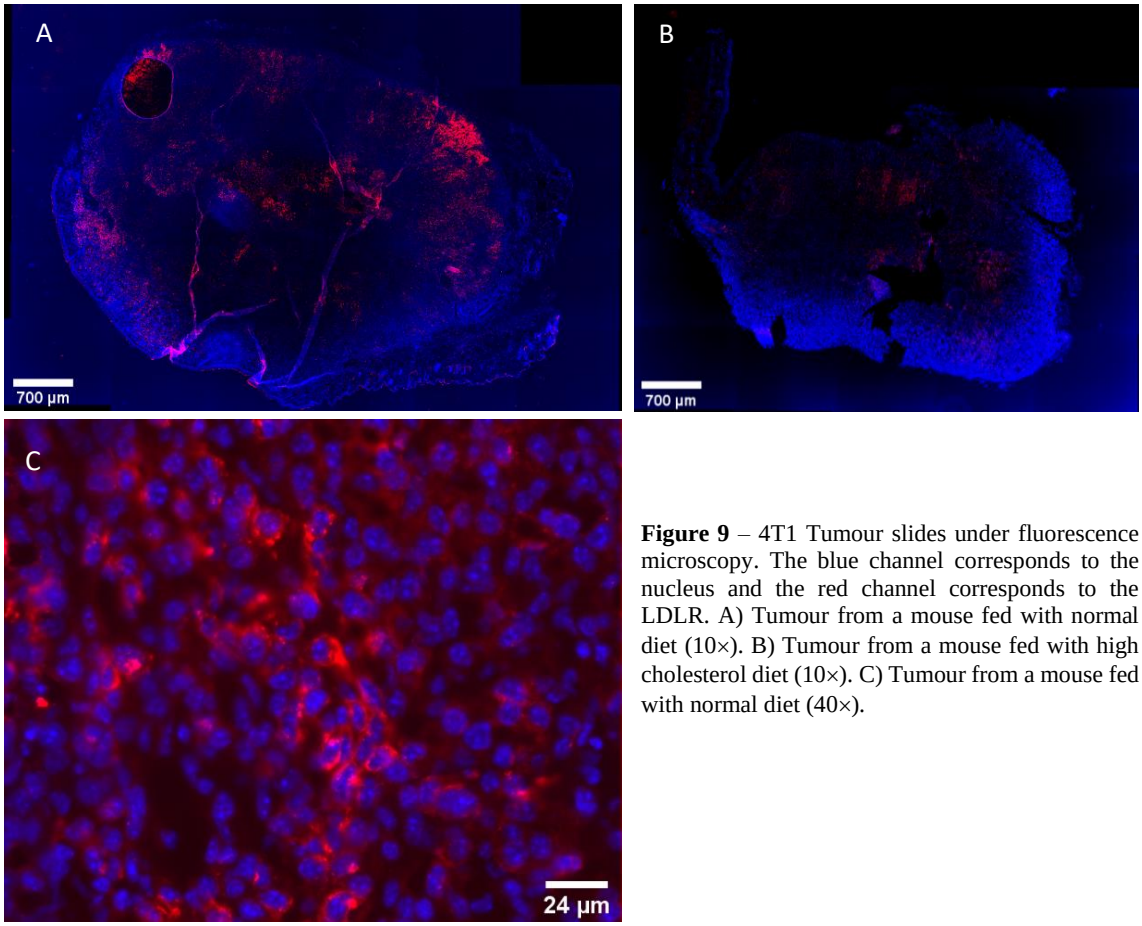


Figure 9 – 4T1 Tumour slides under fluorescence microscopy. The blue channel corresponds to the nucleus and the red channel corresponds to the LDLR. A) Tumour from a mouse fed with normal diet (10×). B) Tumour from a mouse fed with high cholesterol diet (10×). C) Tumour from a mouse fed with normal diet (40×).

The first goal of this experiment was to address the distribution of LDLR across the tumour (Figure 9). We were able to observe that LDLR is heterogeneously distributed across the tumours. In the future, it will be interesting to see whether areas of high tumour cell LDLR expression correlate with areas of increased immune infiltrate and IFNγ production. Also, as expected based on our *in vitro* data, cells can sense a high LDL environment and respond to it through a negative feedback loop that leads to decreased LDLR expression. This also strengths the specificity of the antibody and of the signal we detect.

Discussion and Conclusions

It is becoming increasingly evident that the deregulation of cholesterol metabolism in cancer cells, in particular cholesterol uptake, have consequences for tumour progression [7-11]. How cholesterol uptake is regulated in breast cancer cells, is, however, largely understudied. The LDLR is the main receptor for LDL and, as mentioned LDLR expression is regulated, in non-malignant cells, by cholesterol levels in a negative feedback loop system [16]. Studies performed in other cell types, such as leukemias, prostate and Daudi Burkitt's lymphoma cells, have shown the existence of a defective negative feedback loop in response to external LDL [94-96]. Here we analysed whether this also happens in breast cancer, using two different cell lines. The results obtained show that the exposure to LDL leads to the downregulation of LDLR, from which we conclude that LDLR expression in these cells is regulated through the same sterol-dependent pathway as described in normal cells. This pathway, as already cited, depends on the activation of SREBP by low cholesterol levels, which will then be transported to the nucleus where it induces LDLR transcription [14].

As stated in the introduction, it is known that immune cells present at the TME play an important role in modulating cancer progression [5], however, the way it affects tumour cell cholesterol metabolism is unknown. The data presented in this work suggests that IFNγ promotes LDLR expression in breast cancer cells at the transcriptional level through JAK/STAT1 signal transduction. Regarding IFNγ, little is known about its influence on LDLR expression. It is known that this cytokine preferentially acts as a defense against the tumor, however, new studies have emerged that show a double face of IFNγ, which may at some point promote tumour progression [72]. It is known, that IFNγ can induce lipid deposition in mouse mesangial cells, partially through the upregulation of SREBP-1 expression [97,98]. SREBP-1, in addition to SREBP-2, was also reported as an inducer of increased LDLR expression [99]. It has also been shown that IFNγ inhibits the expression of sortilin-1 in hepatocytes via JAK/STAT [100]. This protein, in turn, is responsible for the secretion of PCSK9 in hepatocytes, which leads to LDLR degradation [101]. Here by western blot, we see a tendency for decreased PCSK9 expression upon treatment with IFNγ treatment, however at the gene level a tendency to increased expression was observed. Even though we used Brefeldin A to block secretion before doing the western blot for PCSK9, considering that PCSK9 is a secreted protein, it would be relevant in the future to analyse the levels of secreted PCSK9 by ELISA. Another way of regulating LDLR through which IFNγ could act could be through the IDOL protein which, like PCSK9, induces LDLR degradation [102]. IFNγ may inhibit IDOL expression which in turn can increase LDLR expression. Regarding the other cytokines TGF-β, IL-1β and TNFα, these cytokines were expected to increase the expression of LDLR based on other studies published in other types of cells [29-31]. Although there was a tendency for both TNFα and IL-1β induce LDLR expression, this showed a strong variability among different experiments and did not result in statistically significant changes as compared to the control situation. As we discovered that IFNγ increased LDLR expression we went to see if this would have functional implications in cells. First, we

found that the IFNγ-mediated upregulation of LDLR led to increased cholesterol uptake. This result is in line with studies carried out in hepatocytes, where it was seen that an overexpression of the *ldlr* gene in the liver induced the storage of cholesterol and intracellular lipids [103], and also with studies carried out in breast cancer cells where overexpression of LDLR facilitated uptake of LDL-cholesterol [32]. After confirming that the IFN-gamma-mediated increase in LDLR leads to increased cholesterol uptake we then questioned whether this would affect tumour cell behaviour such as proliferation rate and migratory capacity. Concerning proliferation what we were able to observe was that first of all, IFNγ seems to induce a cell growth arrest on this cancer cell type [104]. Cell growth arrest is reported to happen as a response to stress or DNA damage. This arrest happens so that the DNA repair machine can have time to repair the damaged DNA to avoid apoptosis. However, if the damage is too intense, the cell switches to apoptosis [105]. There are several molecular mechanisms that regulate the cell cycle, and it was found that IFNγ acts on several of these mechanisms, one of which is through cyclin-dependent kinases (CDKs), which are protein kinases that, when fully activated, can phosphorylate and activate other proteins that allows the cell cycle advance beyond a checkpoint. More specifically, IFNγ inhibits the function of CDK4 and CDK2, thus preventing cell cycle progression [106]. However, when cells were treated with both LDL and IFNγ proliferated faster than cells treated with IFNγ alone or even than control cells, suggesting that exposure to LDL overcomes the cell cycle arrest induced by IFNγ. Whether this is linked to LDLR expression still needs investigation. Nevertheless, this is particularly interesting and may suggest that increased LDLR-mediated LDL uptake may be a mechanism of overcoming cell growth arrests, as it was revealed that LDL-stimulated cell proliferation was associated with significant increases in the expression of proteins that regulate cell cycle progression, such as CDK2 and CDK4, among others [107]. To note that this was observed using oxidized LDL, which is internalized preferentially via CD36 and less via the LDLR. Concerning migration, we were able to see a tendency for IFNγ on its own to induce breast cancer cell migration, even in the absence of LDL in the growth media. It was found that in triple-negative breast cancer, overexpression of Stat1 is associated with increased lymph node invasion and metastasis, where IFNγ also significantly increased the capacity of MDA-231 cells to invade and migrate towards the serum through this JAK/STAT1 pathway [108]. LDL, after 24 hours, also increased the migration rate of breast cancer cells, as would be expected [7, 8]. When added together, LDL plus IFNγ, it could be observed that there was, in tendency, an even bigger increase in the migratory capacity of these cells, perhaps due to two positive forces that together drive this behaviour even further in the cells, but also perhaps due to the increased expression of LDLR caused by IFNγ, that internalizes more LDL. These results did not, however give statistically significant differences, even when comparing control with LDL treated cells. As such, more replicates need to be performed and and the assay further optimized to draw major conclusions. Also, more experiments such as to block the LDLR in order to observe a reversion of effect need to be performed. The same being true for the proliferation study.

This work aimed to look at the regulation of LDLR expression and cholesterol uptake by LDLR. But there are other receptors that promote the internalization of LDL and it would be interesting in the future to look at their regulation as well [109]. In particular CD36, which appears to play an important role in tumour progression [110,111].

In summary, cytokines present in TME are known to play an important role in modulating tumour progression. Within these cytokines we have IFNγ, which is mostly portrayed as an anti-tumour cytokine, involved in protection against cancer. However, several studies have shown the other face of IFNγ, that is, a more pro-tumour behaviour. Here we show that the induction of LDL uptake on tumour cells downstream of IFNγ could be a mechanism of escape from IFNγ cell cycle arrest, which would occur in areas with available LDL. Taken together, we believe our data will contribute to further characterize how cholesterol metabolism is regulated in breast cancer tumours and also increase our knowledge on the action of immunomodulatory cytokines of the TME. This may have consequences to the discovery and adaptation of new and existing anti-cancer therapies.

References

1. Chiang, A. C., & Massagué, J. (2008). Molecular basis of metastasis. New England Journal of Medicine, 359(26), 2814-2823.
2. Gomes, I. A., & Nunes, C. (2020). Analysis of the Breast Cancer Mortality Rate in Portugal Over a Decade: Spatiotemporal Clustering Analysis. Acta medica portuguesa, 33(5), 305-310.
3. World Health Organization (WHO). (2020). Global Health Estimates 2020: Deaths by Cause, Age, Sex, by Country and by Region, 2000-2019. World Health Organization.
4. Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A., & Bray, F. (2021). Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: a cancer journal for clinicians, 71(3), 209-249.
5. Warburg, O. H., & Dickens, F. (1930). The metabolism of tumours: investigations from the Kaiser Wilhelm Institute for Biology. Berlin-Dahlem (London, UK: Arnold Constable).
6. Warburg, O. (1956a). On the origin of cancer cells. Science 123, 309–314.
7. Warburg, O. (1956b). On respiratory impairment in cancer cells. Science 124, 269–270.
8. VR, P. (1958, July). The biochemical approach to the cancer problem. In Federation proceedings (Vol. 17, No. 2, pp. 691-697).
9. Vander Heiden, M. G., Cantley, L. C., & Thompson, C. B. (2009). Understanding the Warburg effect: the metabolic requirements of cell proliferation. science, 324(5930), 1029-1033.
10. Kennedy, K. M., & Dewhirst, M. W. (2010). Tumor metabolism of lactate: the influence and therapeutic potential for MCT and CD147 regulation. Future Oncology, 6(1), 127-148.
11. Feron, O. (2009). Pyruvate into lactate and back: from the Warburg effect to symbiotic energy fuel exchange in cancer cells. Radiotherapy and oncology, 92(3), 329-333.
12. Semenza, G. L. (2008). Tumor metabolism: cancer cells give and take lactate. The Journal of clinical investigation, 118(12), 3835-3837.
13. Currie, E., Schulze, A., Zechner, R., Walther, T. C., & Farese Jr, R. V. (2013). Cellular fatty acid metabolism and cancer. Cell metabolism, 18(2), 153-161.
14. Xie, H., Tang, C. H. A., Song, J. H., Mancuso, A., Del Valle, J. R., Cao, J., ... & Simon, M. C. (2018). IRE1α RNase–dependent lipid homeostasis promotes survival in Myc-transformed cancers. The Journal of clinical investigation, 128(4), 1300-1316.
15. Pavlova, N. N., & Thompson, C. B. (2016). The emerging hallmarks of cancer metabolism. Cell metabolism, 23(1), 27-47.
16. Hegele, R. A. (2009). Plasma lipoproteins: genetic influences and clinical implications. Nature Reviews Genetics, 10(2), 109-121.
17. Calle, E. E., Rodriguez, C., Walker-Thurmond, K., & Thun, M. J. (2003). Overweight, obesity, and mortality from cancer in a prospectively studied cohort of US adults. New England Journal of Medicine, 348(17), 1625-1638.
18. Dos Santos, C. R., Fonseca, I., Dias, S., & De Almeida, J. M. (2014). Plasma level of LDL-cholesterol at diagnosis is a predictor factor of breast tumor progression. BMC cancer, 14(1), 1-10.

19. dos Santos, C. R., Domingues, G., Matias, I., Matos, J., Fonseca, I., de Almeida, J. M., & Dias, S. (2014). LDL-cholesterol signaling induces breast cancer proliferation and invasion. Lipids in health and disease, 13(1), 1-9.
20. Kim, E. J., Choi, M. R., Park, H., Kim, M., Hong, J. E., Lee, J. Y., ... & Park, J. H. Y. (2011). Dietary fat increases solid tumor growth and metastasis of 4T1 murine mammary carcinoma cells and mortality in obesity-resistant BALB/c mice. Breast Cancer Research, 13(4), 1-13.
21. Alikhani, N., Ferguson, R. D., Novosyadlyy, R., Gallagher, E. J., Scheinman, E. J., Yakar, S., & LeRoith, D. (2013). Mammary tumor growth and pulmonary metastasis are enhanced in a hyperlipidemic mouse model. Oncogene, 32(8), 961-967.
22. Llaverias, G., Danilo, C., Mercier, I., Daumer, K., Capozza, F., Williams, T. M., ... & Frank, P. G. (2011). Role of cholesterol in the development and progression of breast cancer. The American journal of pathology, 178(1), 402-412.
23. Gent, J., & Braakman, I. (2004). Low-density lipoprotein receptor structure and folding. Cellular and Molecular Life Sciences CMLS, 61(19), 2461-2470.
24. Sudhof, T. C., Goldstein, J. L., Brown, M. S., & Russell, D. W. (1985). The LDL receptor gene: a mosaic of exons shared with different proteins. Science, 228(4701), 815-822.
25. Wang, X., Sato, R., Brown, M. S., Hua, X., & Goldstein, J. L. (1994). SREBP-1, a membrane-bound transcription factor released by sterol-regulated proteolysis. Cell, 77(1), 53-62.
26. Goldstein, J. L., DeBose-Boyd, R. A., & Brown, M. S. (2006). Protein sensors for membrane sterols. Cell, 124(1), 35-46.
27. Zhang, L., Reue, K., Fong, L. G., Young, S. G., & Tontonoz, P. (2012). Feedback regulation of cholesterol uptake by the LXR–IDOL–LDLR axis. Arteriosclerosis, thrombosis, and vascular biology, 32(11), 2541-2546.
28. Brown, M. S., & Goldstein, J. L. (1997). The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. Cell, 89(3), 331-340.
29. Wilson, G. M., Vasa, M. Z., & Deeley, R. G. (1998). Stabilization and cytoskeletal-association of LDL receptor mRNA are mediated by distinct domains in its 3′ untranslated region. Journal of lipid research, 39(5), 1025-1032.
30. Wilson, G. M., Roberts, E. A., & Deeley, R. G. (1997). Modulation of LDL receptor mRNA stability by phorbol esters in human liver cell culture models. Journal of lipid research, 38(3), 437-446.
31. Yashiro, T., Yokoi, Y., Shimizu, M., Inoue, J., & Sato, R. (2011). Chenodeoxycholic acid stabilization of LDL receptor mRNA depends on 3′-untranslated region and AU-rich element-binding protein. Biochemical and biophysical research communications, 409(2), 155-159.
32. Matoulkova, E., Michalova, E., Vojtesek, B., & Hrstka, R. (2012). The role of the 3'untranslated region in post-transcriptional regulation of protein expression in mammalian cells. RNA biology, 9(5), 563-576.
33. García-Mauriño, S. M., Rivero-Rodríguez, F., Velázquez-Cruz, A., Hernández-Vellisca, M., Díaz-Quintana, A., De la Rosa, M. A., & Díaz-Moreno, I. (2017). RNA binding protein regulation and cross-talk in the control of AU-rich mRNA fate. Frontiers in molecular biosciences, 4, 71.
34. Lagace, T. A. (2014). PCSK9 and LDLR degradation: regulatory mechanisms in circulation and in cells. Current opinion in lipidology, 25(5), 387.

35. Lagace, T. A., Curtis, D. E., Garuti, R., McNutt, M. C., Park, S. W., Prather, H. B., ... & Horton, J. D. (2006). Secreted PCSK9 decreases the number of LDL receptors in hepatocytes and inlivers of parabiotic mice. The Journal of clinical investigation, 116(11), 2995-3005.
36. Li, J., Tumanut, C., Gavigan, J. A., Huang, W. J., Hampton, E. N., Tumanut, R., ... & Harris, J. L. (2007). Secreted PCSK9 promotes LDL receptor degradation independently of proteolytic activity. Biochemical Journal, 406(2), 203-207.
37. Kwon, H. J., Lagace, T. A., McNutt, M. C., Horton, J. D., & Deisenhofer, J. (2008). Molecular basis for LDL receptor recognition by PCSK9. Proceedings of the National Academy of Sciences, 105(6), 1820-1825.
38. Fisher, T. S., Surdo, P. L., Pandit, S., Mattu, M., Santoro, J. C., Wisniewski, D., ... & Sitlani, A. (2007). Effects of pH and low density lipoprotein (LDL) on PCSK9-dependent LDL receptor regulation. Journal of Biological Chemistry, 282(28), 20502-20512.
39. Zhang, D. W., Lagace, T. A., Garuti, R., Zhao, Z., McDonald, M., Horton, J. D., ... & Hobbs, H. H. (2007). Binding of proprotein convertase subtilisin/kexin type 9 to epidermal growth factor-like repeat A of low density lipoprotein receptor decreases receptor recycling and increases degradation. Journal of Biological Chemistry, 282(25), 18602-18612.
40. Grove, R. I., Mazzucco, C., Allegretto, N., Kiener, P. A., Spitalny, G., Radka, S. F., ... & Warr, G. A. (1991). Macrophage-derived factors increase low density lipoprotein uptake and receptor number in cultured human liver cells. Journal of lipid research, 32(12), 1889-1897.
41. Ruan, X. Z., Moorhead, J. F., Tao, J. L., Ma, K. L., Wheeler, D. C., Powis, S. H., & Varghese, Z. (2006). Mechanisms of dysregulation of low-density lipoprotein receptor expression in vascular smooth muscle cells by inflammatory cytokines. Arteriosclerosis, thrombosis, and vascular biology, 26(5), 1150-1155.
42. Ruan, X. Z., Varghese, Z., Fernando, R., & Moorhead, J. F. (1998). Cytokine regulation of low-density lipoprotein receptor gene transcription in human mesangial cells. Nephrology, Dialysis, Transplantation: Official Publication of the European Dialysis and Transplant Association-European Renal Association, 13(6), 1391-1397.
43. Gallagher, E. J., Zelenko, Z., Neel, B. A., Antoniou, I. M., Rajan, L., Kase, N., & LeRoith, D. (2017). Elevated tumor LDLR expression accelerates LDL cholesterol-mediated breast cancer growth in mouse models of hyperlipidemia. Oncogene, 36(46), 6462-6471.
44. Spill, F., Reynolds, D. S., Kamm, R. D., & Zaman, M. H. (2016). Impact of the physical microenvironment on tumor progression and metastasis. Current opinion in biotechnology, 40, 41-48.
45. Quail, D. F., & Joyce, J. A. (2013). Microenvironmental regulation of tumor progression and metastasis. Nature medicine, 19(11), 1423-1437.
46. Balkwill, F., & Mantovani, A. (2001). Inflammation and cancer: back to Virchow?. The lancet, 357(9255), 539-545.
47. Elinav, E., Nowarski, R., Thaiss, C. A., Hu, B., Jin, C., & Flavell, R. A. (2013). Inflammation-induced cancer: crosstalk between tumours, immune cells and microorganisms. Nature Reviews Cancer, 13(11), 759-771.
48. Goswami, S., Sahai, E., Wyckoff, J. B., Cammer, M., Cox, D., Pixley, F. J., ... & Condeelis, J. S. (2005). Macrophages promote the invasion of breast carcinoma cells via a colony-stimulating factor-1/epidermal growth factor paracrine loop. Cancer research, 65(12), 5278-5283.

49. Qian, B., Deng, Y., Im, J. H., Muschel, R. J., Zou, Y., Li, J., ... & Pollard, J. W. (2009). A distinct macrophage population mediates metastatic breast cancer cell extravasation, establishment and growth. PloS one, 4(8), e6562.
50. Mazzoni, A., Bronte, V., Visintin, A., Spitzer, J. H., Apolloni, E., Serafini, P., ... & Segal, D. M. (2002). Myeloid suppressor lines inhibit T cell responses by an NO-dependent mechanism. The Journal of Immunology, 168(2), 689-695.
51. Von Boehmer, H., & Daniel, C. (2013). Therapeutic opportunities for manipulating T Reg cells in autoimmunity and cancer. Nature reviews Drug discovery, 12(1), 51-63.
52. Patil, R. S., Shah, S. U., Shrikhande, S. V., Goel, M., Dikshit, R. P., & Chiplunkar, S. V. (2016). IL17 producing γδT cells induce angiogenesis and are associated with poor survival in gallbladder cancer patients. International journal of cancer, 139(4), 869-881.
53. Wakita, D., Sumida, K., Iwakura, Y., Nishikawa, H., Ohkuri, T., Chamoto, K., ... & Nishimura, T. (2010). Tumor-infiltrating IL-17-producing γδ T cells support the progression of tumor by promoting angiogenesis. European journal of immunology, 40(7), 1927-1937.
54. Yang, J., & Weinberg, R. A. (2008). Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. Developmental cell, 14(6), 818-829.
55. Peng, G., Wang, H. Y., Peng, W., Kiniwa, Y., Seo, K. H., & Wang, R. F. (2007). Tumor-infiltrating γδ T cells suppress T and dendritic cell function via mechanisms controlled by a unique toll-like receptor signaling pathway. Immunity, 27(2), 334-348.
56. Sabbione, F., Gabelloni, M. L., Ernst, G., Gori, M. S., Salamone, G., Oleastro, M., ... & Jancic, C. C. (2014). Neutrophils suppress γδ T-cell function. European journal of immunology, 44(3), 819-830.
57. Nathan, C., & Sporn, M. (1991). Cytokines in context. Journal of Cell Biology, 113(5), 981-986.
58. Dinarello, C. A. (2000). Proinflammatory cytokines. Chest, 118(2), 503-508.
59. Opal, S. M., & DePalo, V. A. (2000). Anti-inflammatory cytokines. Chest, 117(4), 1162-1172.
60. Popivanova, B. K., Kitamura, K., Wu, Y., Kondo, T., Kagaya, T., Kaneko, S., ... & Mukaida, N. (2008). Blocking TNF-α in mice reduces colorectal carcinogenesis associated with chronic colitis. The Journal of clinical investigation, 118(2), 560-570.
61. Yang, H., Bocchetta, M., Kroczynska, B., Elmishad, A. G., Chen, Y., Liu, Z., ... & Carbone, M. (2006). TNF-α inhibits asbestos-induced cytotoxicity via a NF-κB-dependent pathway, a possible mechanism for asbestos-induced oncogenesis. Proceedings of the National Academy of Sciences, 103(27), 10397-10402.
62. Arnott, C. H., Scott, K. A., Moore, R. J., Hewer, A., Phillips, D. H., Parker, P., ... & Owens, D. M. (2002). Tumour necrosis factor-α mediates tumour promotion via a PKCα- and AP-1-dependent pathway. Oncogene, 21(31), 4728-4738.
63. Nakagawa, J., Saio, M., Tamakawa, N., Suwa, T., Frey, A. B., Nonaka, K., ... & Takami, T. (2007). TNF expressed by tumor-associated macrophages, but not microglia, can eliminate glioma. International journal of oncology, 30(4), 803-811.
64. Morishima, N., Mizoguchi, I., Takeda, K., Mizuguchi, J., & Yoshimoto, T. (2009). TGF-β is necessary for induction of IL-23R and Th17 differentiation by IL-6 and IL-23. Biochemical and biophysical research communications, 386(1), 105-110.
65. Cui, W., Fowlis, D. J., Bryson, S., Duffie, E., Ireland, H., Balmain, A., & Akhurst, R. J. (1996). TGFβ1 inhibits the formation of benign skin tumors, but enhances progression to invasive spindle carcinomas in transgenic mice. Cell, 86(4), 531-542.

66. Miller, L. C., Isa, S., Vannier, E., Georgilis, K., Steere, A. C., & Dinarello, C. A. (1992). Live Borrelia burgdorferi preferentially activate interleukin-1 beta gene expression and protein synthesis over the interleukin-1 receptor antagonist. The Journal of clinical investigation, 90(3), 906-912.
67. Croston, G. E., Cao, Z., & Goeddel, D. V. (1995). NF-κB activation by interleukin-1 (IL-1) requires an IL-1 receptor-associated protein kinase activity. Journal of Biological Chemistry, 270(28), 16514-16517.
68. Voronov, E., Shouval, D. S., Krelin, Y., Cagnano, E., Benharroch, D., Iwakura, Y., ... & Apte, R. N. (2003). IL-1 is required for tumor invasiveness and angiogenesis. Proceedings of the National Academy of Sciences, 100(5), 2645-2650.
69. Jordan, W. J., Eskdale, J., Boniotto, M., Lennon, G. P., Peat, J., Campbell, J. D., & Gallagher, G. (2005). Human IL-19 regulates immunity through auto-induction of IL-19 and production of IL-10. European Journal of Immunology, 35(5), 1576-1582.
70. Said, E. A., Dupuy, F. P., Trautmann, L., Zhang, Y., Shi, Y., El-Far, M., ... & Sekaly, R. P. (2010). Programmed death-1–induced interleukin-10 production by monocytes impairs CD4+ T cell activation during HIV infection. Nature medicine, 16(4), 452-459.
71. Dai, Z. J., Wang, X. J., Zhao, Y., Ma, X. B., Kang, H. F., Min, W. L., ... & Liu, X. X. (2014). Effects of interleukin-10 polymorphisms (rs1800896, rs1800871, and rs1800872) on breast cancer risk: evidence from an updated meta-analysis. Genetic testing and molecular biomarkers, 18(6), 439-445.
72. Rallis, K. S., Corrigan, A. E., Dadah, H., George, A. M., Keshwara, S. M., Sideris, M., & Szabados, B. (2021). Cytokine-based cancer immunotherapy: Challenges and opportunities for IL-10. Anticancer research, 41(7), 3247-3252.
73. Zhao, S., Wu, D., Wu, P., Wang, Z., & Huang, J. (2015). Serum IL-10 predicts worse outcome in cancer patients: A meta-analysis. PloS one, 10(10), e0139598.
74. Changkija, B., & Konwar, R. (2012). Role of interleukin-10 in breast cancer. Breast cancer research and treatment, 133(1), 11-21.
75. Visco, C., Vassilakopoulos, T. P., Kliche, K. O., Nadali, G., Viviani, S., Bonfante, V., ... & Sarris, A. H. (2004). Elevated serum levels of IL-10 are associated with inferior progression-free survival in patients with Hodgkin's disease treated with radiotherapy. Leukemia & lymphoma, 45(10), 2085-2092.
76. Moore, K. W., de Waal Malefyt, R., Coffman, R. L., & O'Garra, A. (2001). Interleukin-10 and the interleukin-10 receptor. Annual review of immunology, 19(1), 683-765.
77. Llanes-Fernández, L., Álvarez-Goyanes, R. I., del Carmen Arango-Prado, M., Alcocer-González, J. M., Mojarrieta, J. C., Pérez, X. E., ... & Rodríguez-Padilla, C. (2006). Relationship between IL-10 and tumour markers in breast cancer patients. The Breast, 15(4), 482-489.
78. Vinod, C., & Jyothy, A. (2015). A common SNP of IL-10 (-1082A/G) is associated with increased risk of premenopausal breast cancer in South Indian women. Iranian journal of cancer prevention, 8(4).
79. Howell, M. W. (2013). Interleukin-10 gene polymorphisms and cancer. In Madame Curie Bioscience Database [Internet]. Landes Bioscience.
80. Burke, J. D., & Young, H. A. (2019, June). IFN-γ: a cytokine at the right time, is in the right place. In Seminars in immunology (Vol. 43, p. 101280). Academic Press.
81. Dunn, G. P., Koebel, C. M., & Schreiber, R. D. (2006). Interferons, immunity and cancer immunoediting. Nature Reviews Immunology, 6(11), 836-848.
82. Jorgovanovic, D., Song, M., Wang, L., & Zhang, Y. (2020). Roles of IFN-γ in tumour progression and regression: A review. Biomarker Research, 8(1), 1-16.

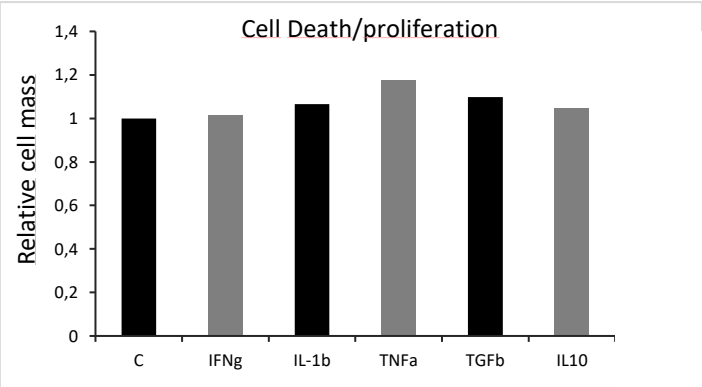
83. Zaidi, M. R., & Merlino, G. (2011). The two faces of interferon-γ in cancer. Clinical cancer research, 17(19), 6118-6124.
84. Song, M., Ping, Y., Zhang, K., Yang, L., Li, F., Zhang, C., ... & Zhang, Y. (2019). Low-dose IFNγ induces tumor cell stemness in tumor microenvironment of non–small cell lung cancer. Cancer research, 79(14), 3737-3748.
85. Kelly, S. A., Gschmeissner, S., East, N., & Balkwill, F. R. (1991). Enhancement of metastatic potential by γ-interferon. Cancer research, 51(15), 4020-4027.
86. Chen, H. C., Chou, A. S. B., Liu, Y. C., Hsieh, C. H., Kang, C. C., Pang, S. T., ... & Liao, S. K. (2011). Induction of metastatic cancer stem cells from the NK/LAK-resistant floating, but not adherent, subset of the UP-LN1 carcinoma cell line by IFN-γ. Laboratory Investigation, 91(10), 1502-1513.
87. Singh, S., Kumar, S., Srivastava, R. K., Nandi, A., Thacker, G., Murali, H., ... & Chakrabarti, R. (2020). Loss of ELF5–FBXW7 stabilizes IFNGR1 to promote the growth and metastasis of triple-negative breast cancer through interferon-γ signalling. Nature cell biology, 22(5), 591-602.
88. Lane, R. S., Femel, J., Breazeale, A. P., Loo, C. P., Thibault, G., Kaempf, A., ... & Lund, A. W. (2018). IFNγ-activated dermal lymphatic vessels inhibit cytotoxic T cells in melanoma and inflamed skin. Journal of Experimental Medicine, 215(12), 3057-3074.
89. Pai, C. C. S., Huang, J. T., Lu, X., Simons, D. M., Park, C., Chang, A., ... & Fong, L. (2019). Clonal deletion of tumor-specific T cells by interferon-γ confers therapeutic resistance to combination immune checkpoint blockade. Immunity, 50(2), 477-492.
90. He, Y. F., Wang, X. H., Zhang, G. M., Chen, H. T., Zhang, H., & Feng, Z. H. (2005). Sustained low-level expression of interferon-γ promotes tumor development: potential insights in tumor prevention and tumor immunotherapy. Cancer Immunology, Immunotherapy, 54(9), 891-897.
91. Schectman, G., Kaul, S., Mueller, R. A., Borden, E. C., & Kissebah, A. H. (1992). The effect of interferon on the metabolism of LDLs. Arteriosclerosis and thrombosis: a journal of vascular biology, 12(9), 1053-1062.
92. Reiss, A. B., Awadallah, N. W., Malhotra, S., Montesinos, M. C., Chan, E. S., Javitt, N. B., & Cronstein, B. N. (2001). Immune complexes and IFN-γ decrease cholesterol 27-hydroxylase in human arterial endothelium and macrophages. Journal of Lipid Research, 42(11), 1913-1922.
93. Reiss, A. B., Patel, C. A., Rahman, M. M., Chan, E. S., Hasneen, K., Montesinos, M. C., ... & Cronstein, B. N. (2004). Interferon-γ impedes reverse cholesterol transport and promotes foam cell transformation in THP-1 human monocytes/macrophages. Medical Science Monitor, 10(11), BR420-BR425.
94. Sankanagoudar, S., Singh, G., Mahapatra, M., Kumar, L., & Chandra, N. C. (2017). Cholesterol homeostasis in isolated lymphocytes: A differential correlation between male control and chronic lymphocytic leukemia subjects. Asian Pacific journal of cancer prevention: APJCP, 18(1), 23.
95. Chen, Y., & Hughes-Fulford, M. (2001). Human prostate cancer cells lack feedback regulation of low-density lipoprotein receptor and its regulator, SREBP2. International journal of cancer, 91(1), 41-45.
96. Yen, C. F., Kalunta, C. I., Chen, F. S., Kaptein, J. S., Lin, C. K. E., & Lad, P. M. (1995). Regulation of low-density lipoprotein receptors and assessment of their functional role in Burkitt's lymphoma cells. Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism, 1257(1), 47-57.

97. Zhang, Y. J., Chen, Y. N., Zheng, S. S., Liu, Q. J., Hao, J., Han, Y., ... & Liu, S. X. (2013). HMGB1/SREBP-1 mediated IFN-gamma-induced lipid deposition in mouse mesangial cells. Zhongguo ying yong sheng li xue za zhi= Zhongguo yingyong shenglixue zazhi= Chinese journal of applied physiology, 29(1), 6-10.
98. Hao, J., Zhang, Y. J., Lv, X., Xu, N., Liu, Q. J., Zhao, S., ... & Liu, S. X. (2013). IFN-γ induces lipogenesis in mouse mesangial cells via the JAK2/STAT1 pathway. American Journal of Physiology-Cell Physiology, 304(8), C760-C767.
99. Guo, D., Reinitz, F., Youssef, M., Hong, C., Nathanson, D., Akhavan, D., ... & Mischel, P. S. (2011). An LXR agonist promotes glioblastoma cell death through inhibition of an EGFR/AKT/SREBP-1/LDLR–dependent pathway. Cancer discovery, 1(5), 442-456.
100. Pirault, J., Polyzos, K. A., Petri, M. H., Ketelhuth, D. F., Bäck, M., & Hansson, G. K. (2017). The inflammatory cytokine interferon-gamma inhibits sortilin-1 expression in hepatocytes via the JAK/STAT pathway. European journal of immunology, 47(11), 1918-1924.
101. Gustafsen, C., Kjolby, M., Nyegaard, M., Mattheisen, M., Lundhede, J., Buttenschøn, H., ... & Glerup, S. (2014). The hypercholesterolemia-risk gene SORT1 facilitates PCSK9 secretion. Cell metabolism, 19(2), 310-318.
102. Zelcer, N., Hong, C., Boyadjian, R., & Tontonoz, P. (2009). LXR regulates cholesterol uptake through Idol-dependent ubiquitination of the LDL receptor. Science, 325(5936), 100-104.
103. Cichon, G., Willnow, T., Herwig, S., Uckert, W., Löser, P., Schmidt, H. H., ... & Heeren, J. (2004). Non-physiological overexpression of the low density lipoprotein receptor (LDLr) gene in the liver induces pathological intracellular lipid and cholesterol storage. The Journal of Gene Medicine: A cross-disciplinary journal for research on the science of gene transfer and its clinical applications, 6(2), 166-175.
104. Doherty, G. M., Tsung, K., McCluskey, B., & Norton, J. A. (1996). Endogenous Interferon-γ Acts Directly on Tumour Cellsin Vivoto Suppress Growth. Journal of Surgical Research, 64(1), 68-74.
105. Johnson, D. G., & Walker, C. L. (1999). Cyclins and cell cycle checkpoints. Annual review of pharmacology and toxicology, 39.
106. Sangfelt, O., Erickson, S., & Grandér, D. (2000). Mechanisms of interferon-induced cell cycle arrest. Front Biosci, 5, D479-87.
107. Zettler, M. E., Prociuk, M. A., Austria, J. A., Massaeli, H., Zhong, G., & Pierce, G. N. (2003). OxLDL stimulates cell proliferation through a general induction of cell cycle proteins. American Journal of Physiology-Heart and Circulatory Physiology, 284(2), H644-H653.
108. Greenwood, C., Metodieva, G., Al-Janabi, K., Lausen, B., Alldridge, L., Leng, L., ... & Metodiev, M. V. (2012). Stat1 and CD74 overexpression is co-dependent and linked to increased invasion and lymph node metastasis in triple-negative breast cancer. Journal of proteomics, 75(10), 3031-3040.
109. Luo, C., Wang, D., Huang, W., Song, Y., Ge, L., Zhang, X., ... & Wang, Q. (2021). Feedback regulation of coronary artery disease susceptibility gene ADTRP and LDL receptors LDLR/CD36/LOX-1 in endothelia cell functions involved in atherosclerosis. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 1867(7), 166130.
110. Yoshida, T., Yokobori, T., Saito, H., Kuriyama, K., Kumakura, Y., Honjo, H., ... & Shirabe, K. (2021). CD36 expression is associated with cancer aggressiveness and

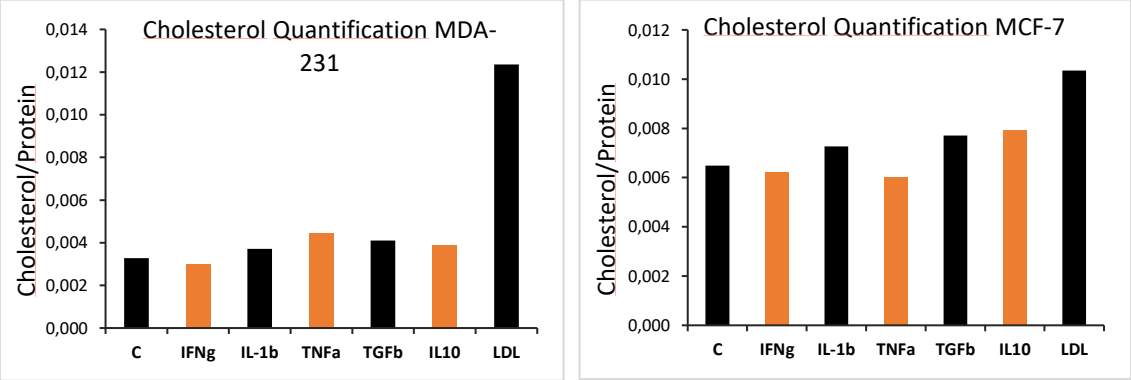
energy source in esophageal squamous cell carcinoma. Annals of Surgical Oncology, 28(2), 1217-1227.

111. Ma, X., Xiao, L., Liu, L., Ye, L., Su, P., Bi, E., ... & Yi, Q. (2021). CD36-mediated ferroptosis dampens intratumoral CD8+ T cell effector function and impairs their antitumour ability. Cell Metabolism, 33(5), 1001-1012.

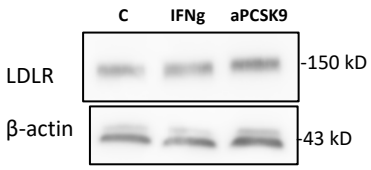
Supplementary data

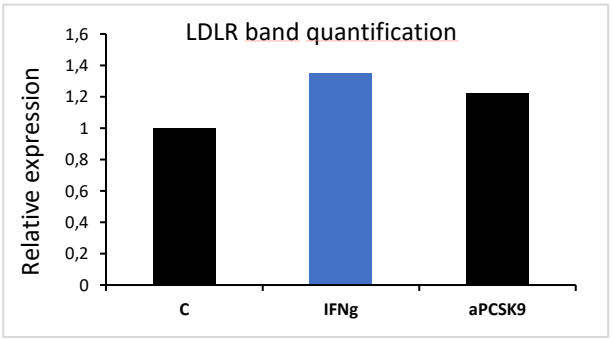


Supplementary figure 1- Sulforhodamine B assay for MDA-231. Cells were treated with different cytokines for 24h. The reletive number of cell mass were determined by fluorescence intensity. n=1.

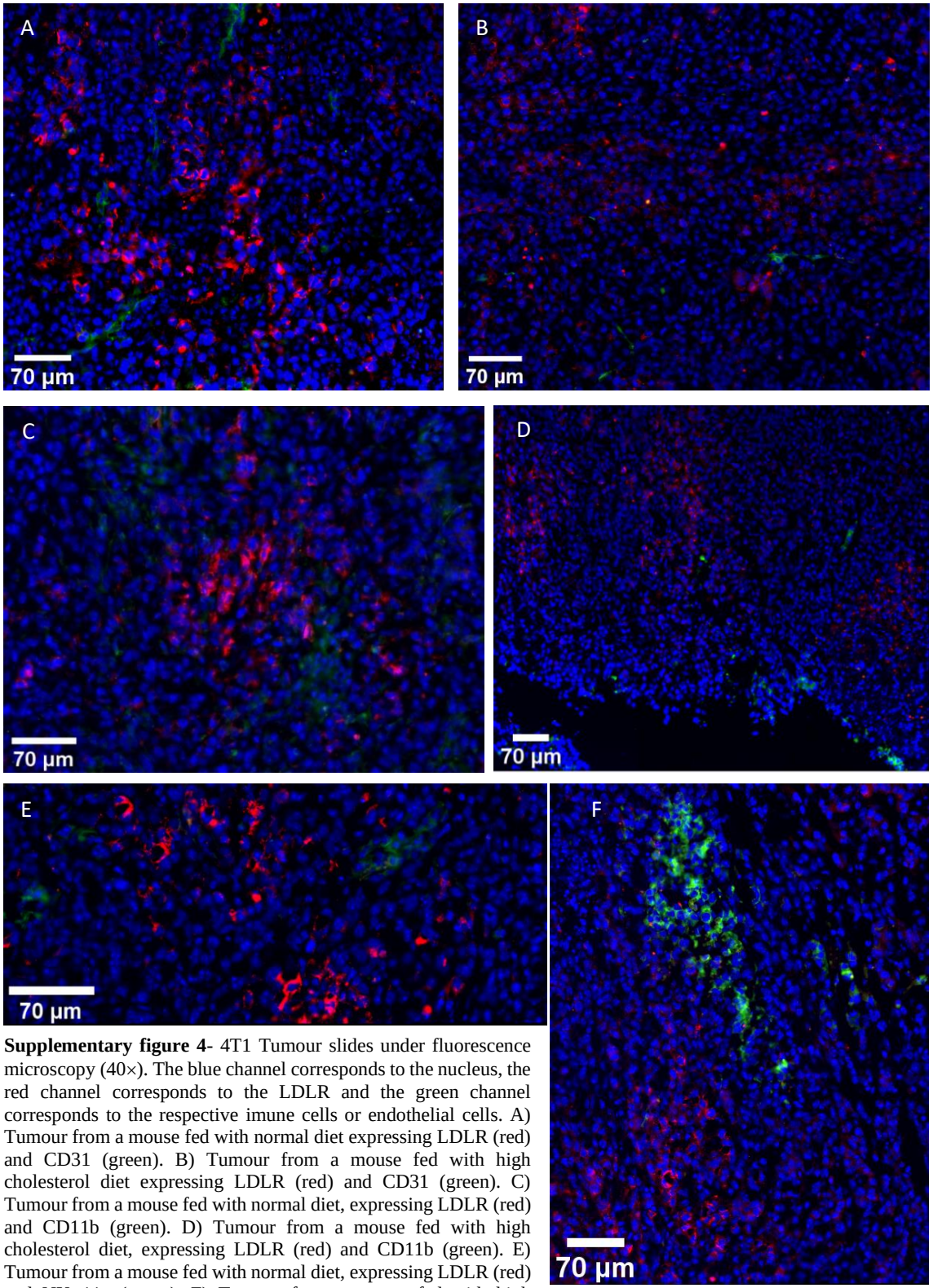


Supplementary figure 2– Amplex red assay. (Left) Cholesterol quantification in breast cancer cells (MDA-231 cell line). (Right) Cholesterol quantification in breast cancer cells (MCF-7 cell line). Both cell lines were treated with LDL or diferent cytokines for 24h. n=1





Supplementary figure 3– Immunoblot bands and their respective relative LDLR band quantification for MDA-231 cell line, treated with IFNγ or αPCSK9 for 24h. The upper band in the immunoblot corresponds to LDLR while the lower band corresponds to β-actin. The values determined in the band intensity quantification correspond to the relative LDLR band intensity values (which are calculated by the ratio between the intensities of the LDLR band and the β-actin band) in comparison with the control band's relative intensity. n=1



Supplementary figure 4- 4T1 Tumour slides under fluorescence microscopy (40×). The blue channel corresponds to the nucleus, the red channel corresponds to the LDLR and the green channel corresponds to the respective imune cells or endothelial cells. A) Tumour from a mouse fed with normal diet expressing LDLR (red) and CD31 (green). B) Tumour from a mouse fed with high cholesterol diet expressing LDLR (red) and CD31 (green). C) Tumour from a mouse fed with normal diet, expressing LDLR (red) and CD11b (green). D) Tumour from a mouse fed with high cholesterol diet, expressing LDLR (red) and CD11b (green). E) Tumour from a mouse fed with normal diet, expressing LDLR (red) and NKp44+ (green). F) Tumour from a mouse fed with high cholesterol diet, expressing LDLR (red) and NKp44+ (green).