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UNIVERSIDAD AUTÓNOMA METROPOLITANA

Universidad Autónoma Metropolitana
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El papel del factor de crecimiento y diferenciación 11 en la polarización de macrófagos y su implicación en el carcinoma hepatocelular

The role of growth differentiation factor 11 on macrophage polarization and its implications in hepatocellular carcinoma

TESIS:

Que para obtener el grado de Maestro en Biología Experimental

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3. ABSTRACT

The growth differentiation factor 11 (GDF11) promotes the loss of an aggressive phenotype in various tumors, which has been studied in our research group using cell lines derived from hepatocellular carcinoma (HCC). Due to this loss in aggressiveness directly in transformed cells, in the present work, we focus on the study of the tumor microenvironment (TME), specifically macrophages with immunosuppressive and anti-inflammatory characteristics (M2) from which the tumor obtains advantages such as proliferation, invasion, and cell migration. For this, macrophages derived from the THP-1 cell line differentiated with PMA (200 ng / mL) were cultured, which responded to the activation mediated by GDF11 (50 ng / mL) observing phosphorylation of the Smad2 / 3 proteins at 5 minutes. , indicating that these cells respond to signaling mediated by this molecule and that it may also be involved in polarity. Furthermore, it was found that GDF11 ligand does not alter cell viability or proliferation.

Macrophage activation was analyzed using LPS (50 ng/mL) and anti-inflammatory interleukins (IL-4/13) observing the acquisition of their M1 and M2 polarity, respectively, by flow cytometry. On the other hand, different polarization methods were carried out by using conditioned media (CM) derived from HCC cell lines in which macrophages obtain a more efficient M2 polarization, observed with the presence of the CD206 marker. Once the M2 polarity was standardized, the macrophages were treated with GDF11 in competition with IL-4/13, observing that GDF11 induces a decrease in the percentage of CD206 + cells, indicating that this molecule is involved in re-polarization

and that it also suggests a reduction in aggressiveness in liver tumors. On the other hand, it was observed that GDF11 is involved in lipid metabolism, since a decrease in total cholesterol levels was detected, verifying this same result with the use of statins, mainly atorvastatin (10 μM). The results indicate that GDF11 indirectly modulates the mevalonate pathway and cholesterol synthesis, affecting the polarity of M2 macrophages, suggesting that the tumor microenvironment (TME) is also affected and that it may have repercussions, reducing the aggressiveness of the tumor.

4. RESUMEN

Introducción y antecedentes

El carcinoma hepatocelular (HCC) representa un serio problema a nivel mundial, abarcando mas del 80% de los tumores primarios, donde se estima que representa el cuarto lugar en mortalidad. El HCC tiene diferentes etiologías incluyendo infecciones virales como la hepatitis B y C, xenobióticos, alto consumo de alcohol y de lípidos como el colesterol. El colesterol promueve la formación de tumores con una elevada agresividad, grandes y altamente vascularizados. El colesterol puede ser obtenido de la dieta o mediante el incremento de la síntesis *de novo* por la ruta del mevalonato promovido por la enzima limitante HMG-CoA reductasa. En el proceso del desarrollo tumoral ocurre una inflamación crónica, también llamada inflamación no resuelta, la cual promueve el reclutamiento de leucocitos, que forman parte del microambiente tumoral (TME). Estas células del sistema inmunológico forman un tipo específico de TME llamado como microambiente inmunológico tumoral (TIME). Dentro del TIME las células que mas abundan son los macrófagos, y que también son llamados macrófagos asociados a tumores (TAM) con una activación o polaridad alternativa (M2) con respuesta pro-tumoral, es decir, le brindan agresividad al tumor. Los TAM son sometidos a un ambiente anti-inflamatorio y ejercen la inmunosupresión de otros leucocitos. Moléculas como el TGF-β son secretadas por el tumor promoviendo una regulacion autocrina y/o paracrina activando M2-TAM, los cuales son identificados mediante marcadores especificos como lo son CD163 y CD206.

El uso de moléculas que puedan revertir la polaridad de los TAM y que además ataquen a las células transformadas representaría una nueva estrategia para el tratamiento de diversos tumores, incluyendo el HCC.

Se ha estudiado el papel del factor de crecimiento y diferenciación 11 en células derivadas de HCC donde disminuye su efecto de agresividad ya mencionados y que además tiende a realizar los efectos opuestos al TGF-β, como la disminución en la síntesis de colesterol y la migración celular. Por lo que se propone a esta molécula como una molécula capaz de activar macrófagos con una respuesta anti-tumoral (M1). Cabe mencionar que el uso de estatinas también promueven la disminución del colesterol mediante la inhibición de la enzima limitante HMG-CoA reductasa y promoviendo la polarización de macrófagos en diferentes modelos experimentales. El uso de ambas moléculas representaría una terapia combinada con una elevada respuesta.

Pregunta de investigación

¿El tratamiento con GDF11 afectará la polaridad de los TAM y su intercomunicación con células derivadas de HCC?

Hipótesis

El tratamiento con GDF11 modificará la polaridad de los TAM reduciendo el fenotipo agresivo de células derivadas de HCC.

Objetivo general

Evaluar la polaridad de los TAM y su papel en la intercomunicación con células derivadas de un HCC durante el tratamiento con GDF11.

Objetivos específicos

- Caracterizar la vía de señalización mediada por proteínas Smad en la polaridad de macrófagos en presencia de GDF11.
- Evaluar y comparar la polaridad de los macrófagos utilizando medios acondicionados de líneas derivadas de HCC e interleucinas antiinflamatorias.
- Evaluar la polaridad de los TAM en presencia de GDF11.

Materiales y métodos

Para el desarrollo de los objetivos mencionados se utilizó la línea celular de monocitos THP-1, las cuales se diferenciaron en macrófagos mediante el uso de PMA (200 ng/mL) y la línea celular derivada de carcinoma hepatocelular Huh-7.

Los macrófagos fueron tratados en presencia de GDF11 (50 ng/mL) y se evaluó la activación de las proteínas Smad mediante la técnica de western blot. Posteriormente se evaluó la viabilidad y el número de células con el uso del colorante cristal violeta en presencia de este GDF11 o atorvastatina (10 µM).

Las diferentes polaridades M1 y M2 en macrófagos fueron evaluadas mediante el uso de la citometría de flujo y western blot. Para macrófagos M1 (LPS 50 ng/mL) y M2 (IL-4,13 20 ng/mL o medios condicionados de células Huh-7) se utilizaron los marcadores

CD86 y CD206, respectivamente. Mediante el uso de oftaldehido (OPA) se realizó la cuantificación de colesterol total en macrófagos tratados con GDF11 o atorvastatina. Se evaluaron las morfologías de las células con diferentes tratamientos mediante el uso de microscopia de campo claro. Se utilizó microscopia confocal para observar el contenido de CD206 en macrófagos M2 mediante inmunofluorescencias (IF).

Resultados

El factor de crecimiento y diferenciación 11 (GDF11) promueve la perdida de un fenotipo agresivo en diversos tumores, mismo que se ha estudiado en nuestro grupo de investigación utilizando líneas celulares derivadas de carcinoma hepatocelular (HCC). Debido a esta perdida en la agresividad directamente en células transformadas, en el presente trabajo nos enfocamos en el estudio del microambiente tumoral (TME), específicamente en macrófagos con características inmunosupresoras y anti-inflamatorias (M2) de los cuales el tumor obtiene ventajas como proliferación, invasión y migración celular. Para esto se cultivaron macrófagos derivados de la línea celular THP-1 diferenciados con PMA (200 ng/mL), los cuales respondieron a la activación mediada por el GDF11 (50 ng/mL) observando fosforilación de las proteínas Smad2/3 a 5 minutos, indicando que estas células responden a la señalización mediada por esta molécula y que además puede estar involucrada en la polaridad. Además se encontró que el ligando GDF11 no altera la viabilidad ni la proliferación celular.

Se analizó la activación de macrófagos utilizando LPS (50 ng/mL) e interleucinas anti-inflamatorias (IL-4/13) observando la adquisición de su polaridad M1 y M2, respectivamente, por citometría de flujo. Por otro lado, diferentes métodos de polarización se llevaron a cabo mediante el uso de medios condicionados (CM) derivados de líneas celulares de HCC en el cual los macrófagos obtienen una polarización M2 mas eficaz, observada con la presencia del marcador CD206. Una vez estandarizada la polaridad M2, los macrófagos fueron tratados con GDF11 en competencia con IL-4/13 observando que el GDF11 induce un descenso en el porcentaje de células CD206+, indicando que esta molécula está implicada en la re-polarización y que además sugiere una reducción en la agresividad en los tumores hepáticos. Por otro lado, se observó que el GDF11 esta implicado en el metabolismo lipídico, ya que se detectó un descenso en los niveles totales de colesterol, comprobando este mismo resultado con el uso de estatinas, principalmente, atorvastatina (10 µM). Los resultados indican que el GDF11 modula de forma indirecta la ruta del mevalonato y la síntesis de colesterol repercutiendo en la polaridad de macrófagos M2, sugiriendo que el microambiente tumoral (TME) también es afectado y que puede repercutir disminuyendo la agresividad del tumor.

Conclusiones

El ligando GDF11 se postula como una molécula que puede atacar directamente el tumor y las células del microambiente tumoral como los macrófagos involucrando su polaridad.

5. INTRODUCTION AND BACKGROUND

5.1 Epidemiology of hepatocellular carcinoma

Hepatocellular carcinoma (HCC) represents a serious problem worldwide, encompassing more than 80% of primary cancer tumors, it is estimated to represent the fourth most common causes of cancer-related death (J. D. Yang et al., 2019). Liver tumors are ranked sixth in incidence and fourth in mortality. The world health organization has estimated that by the year 2030 more than a million people will die from this type of tumor. Liver cancer is the second most lethal tumor, after pancreatic cancer (Villanueva, 2019).

HCC has a higher prevalence may vary depending on the region (El- Serag, 2020). In countries like Japan, North America and Europe, the age of onset is over 60 years. On the contrary, in Asian countries and most African countries, this type of tumor is diagnosed between the ages of 30 and 60, which means that it is prevalent in those regions of the world (Figure 1). In Mexico, liver tumors are located in ninth place, affecting men and women equally, however, it represents the third place in mortality, which means that it is a highly aggressive tumor (https://gco.iarc.fr/today/home).

HCC has different etiologies, including infections by hepatitis B and C viruses, xenobiotics, high intake of alcohol and lipids such as cholesterol (J. D. Yang et al., 2019).

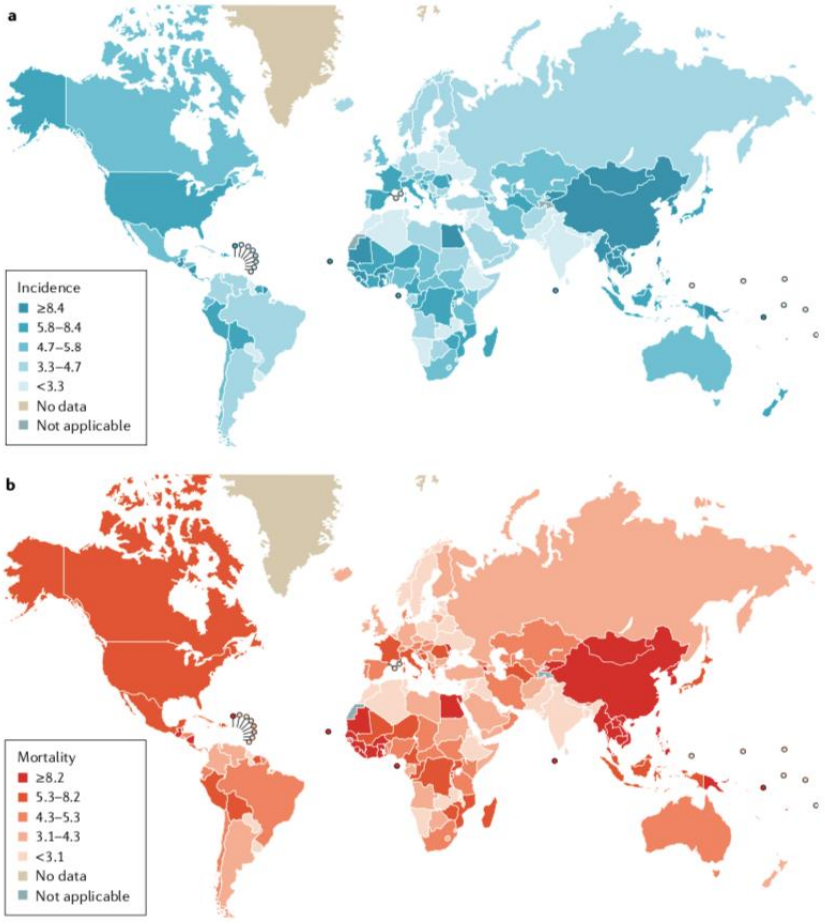


Figure 1. *Global representation of the incidence and mortality of liver tumors. The numbers are per 100,000 people a year. Information obtained from Globocan 2018 (https://gco.iarc.fr/today/home).*

It has been widely described that cholesterol promotes the formation of highly aggressive liver tumors, that is, larger and highly vascularized tumors(Enríquez-Cortina et al., 2017). Regardless of the intake of cholesterol in the diet, the tumor cell increases the synthesis of this molecule through the mevalonate pathway, where, in addition, products such as geranyls and isoprenoids are generated, maintaining active various oncogenic signaling pathways (Xue et al., 2020).

Tumor cells benefit from cholesterol from diet or de novo synthesis and they also obtain it from other cells that are manipulated in their tumor microenvironment (TME). One of these manipulated cell types are macrophages, which can carry out the secretion of cholesterol to maintain the aggressive phenotype of the tumor cell (Goossens et al., 2019).

5.2 Unresolved inflammation and the tumor immunological microenvironment

Chronic inflammation occurs in HCC, which is also called "unresolved inflammation", which is promoted by the activation of key transcription factors such as NF-kB and STAT proteins, both in transformed cells and immune cells which together promote the development of this pathology (Yu et al., 2018).

Within this inflammation, there is a specific type of TME, known as the tumor immune microenvironment (TIME) observed in figure 2, which involves the recruitment of

various immune cells of myeloid and lymphoid origin, where each line can carry out a specific answer that it can eliminate or benefit the growth of the tumor mass, various studies clarify that macrophages are responsible for most of the acquisition of this aggressiveness (Binnewies et al., 2018).

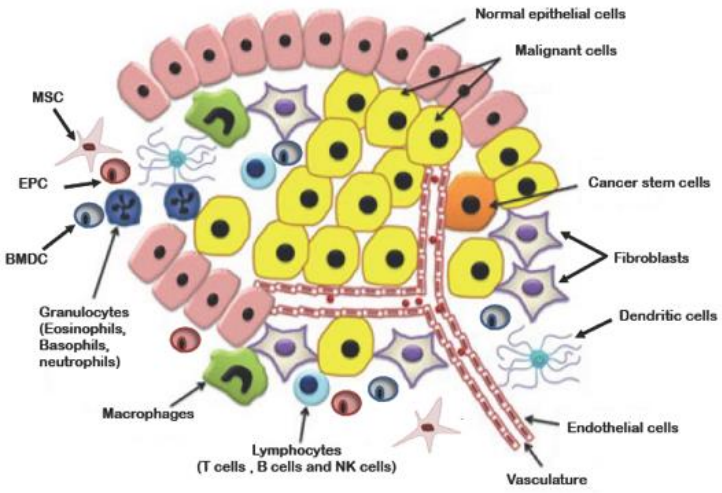
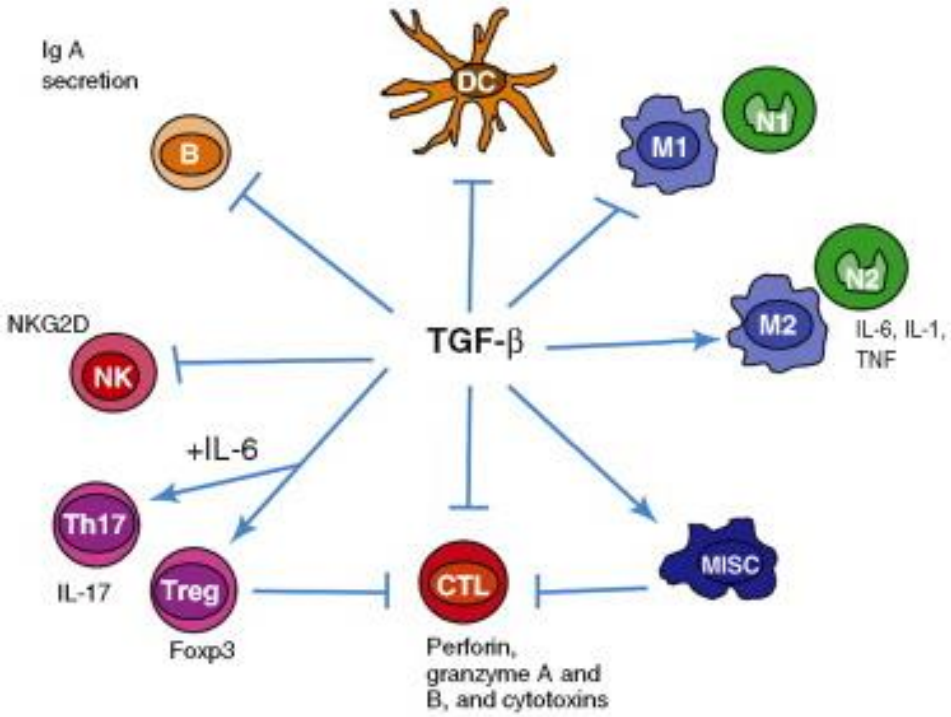


Figure 2. *Diagram of the tumor microenvironment (TME). Illustration of the TME in which immunological cells are involved representing TIME. Image obtained from* (Upreti et al., 2013)

5.3 Tumor-associated macrophages (TAM)

Macrophages are cells belonging to innate immunity with high plasticity responding according to the environmental conditions. The primary function is the elimination of pathogens through the process of phagocytosis or tissue homeostasis. Macrophages play an very important role in many pathologies, where in the case of neoplasms they are called tumor associated macrophages (TAM) (Tian et al., 2019).

Macrophages can have different polarities, called M1 or M2. The TAM present in TIME tends to be activated alternately, they acquire an M2 polarity which presents pro-tumor responses, and the tumor has benefit of this polarity (Bao et al., 2019). Unlike classically activated macrophages or macrophages with M1 polarity (anti-tumor response), M2-TAM tends to secrete anti-inflammatory interleukins (IL) in addition to immunosuppressive molecules, as is the case of TGF-β and low production of pro-inflammatory molecules, which together promote the growth of different types of tumors, including HCC (Zhang et al., 2016b). TGF-β secreted by the tumor and the TME tends to activate a great variety of leukocytes that would give the tumor cells aggressive characteristics, promoting the M2 polarity by the TAM as shown in figure 3 (L. Yang et al., 2010).



TGF-β regulation of immune cells in tumor microenvironment

Figure 3. *The TGF-β molecule promotes effective immunosuppression in TIME, in addition to favoring M2 polarity in macrophages. Image obtained from (*(L. Yang et al., 2010).

TAMs with M2 polarity (M2-TAM) benefit from a wide variety of responses including anti-inflammatory and immunosuppressive responses promoted by Th2 lymphocytes and the tumor. This is supported by the evidence of a change in the secretion of Th1 cytokines (IL-2, TNF-a and IFN-Y) by Th2 (IL-4, IL-6 and IL-10), in addition to finding high levels of alpha -fetoprotein (AFP) in plasma(Ji et al., s/f). The anti-inflammatory interleukins IL-4 and IL-13 promote M2 polarization of macrophages(Bhattacharjee et al., 2013; Orecchioni et al., 2019). It is important to mention that through in-vitro studies macrophages are differentiated with the use of these interleukins, while in-vivo, they are differentiated by tumor secretions that include CSF1, Wnt, IL-8, HMGB1, among others such as is shown in figure 4 (Li et al., 2019).

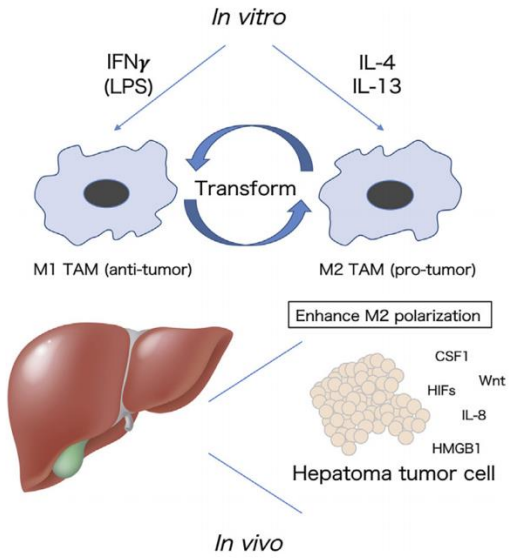


Figure 4. *Macrophage polarization in-vitro by using IL-4/13 or in-vivo by tissue secretions and tumor cells. Image obtained from* (Li et al., 2019)*.*

M2 macrophages are easily identified by the CD206 or CD163 marker, this has been observed in patient samples, it also represents a poor prognostic marker (Guo et al., 2017). On the other hand, CD80 and CD86 are macrophage markers with M1 polarity, which represent a favorable prognosis (Sun et al., 2020). Recent studies indicate an increase in CD206 due to the effect of the TGF-β molecule (Zhang et al., 2016b).

In murine models, it was observed that in ovarian and breast tumors, M2-TAMs promote the formation of cholesterol by activating the mevalonate pathway and its responsible enzymes (Goossens et al., 2019; Mira et al., 2013). The use of molecules such as statins block the formation of mevalonate, by inhibiting the enzyme 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (HMG-CoA-R), which also results in the reduction of the synthesis of cholesterol and its derivatives, providing greater survival in patients with HCC (Thrift et al., 2019). It has been described in animal models of breast cancer that inhibition of the mevalonate pathway can promote the loss of the pro-tumor response of macrophages (Mira et al., 2013). It has also been reported that the use of statins in combination with other drugs increases their efficacy, which is reflected in the survival of patients, that is, it decreases mortality (Omori et al., 2019).

5.4 Therapies against TAM and HCC

Various immunotherapies have been reported that use monoclonal antibodies against the checkpoints of the immune system, for example, PD1, PD-L1, and CTLA-4, or through the use of drugs that promote the inhibition of specific signaling pathways that involve the proliferation of transformed cells (Kudo, 2017). There are other therapies that target TAMs, such as: 1) inhibition of monocyte recruitment; 2) elimination of TAM within tumor tissue; 3) neutralization of released products; and 4) the re-education or repolarization of macrophages, going from an M2 polarity to an M1, pro-tumor and anti-tumor, respectively (Tian et al., 2019). This last strategy is of great interest since it could be the most important therapy against TAM.

In recent years, they have been looking for molecules that promote this anti-tumor polarity by M2-TAM or their elimination and that can, indirectly, reduce the aggressiveness of the tumor. An example of these molecules is the peptides that activate the immune response against CD206, which promotes cell death and macrophage repolarization (Jaynes et al., 2020). On the other hand, molecules with an effect contrary to TGF-β or anti-inflammatory cytokines would be implicated in the re-education of macrophages, hence the importance of their study.

5.5 Growth differentiation factor 11

The growth differentiation factor 11 (GDF11) has been postulated as a molecule with an antitumor response, despite being part of the TGF-β family (Simoni-Nieves et al., 2019). GDF11 promotes a decrease in the aggressive phenotype in different HCC cell

lines such as Huh-7 and Hep-3B. By activating the Smad 2/3 proteins by phosphorylation, GDF11 reduces proliferation, invasion and migration processes, in addition to promoting a mesenchymal-epithelial transition (MET) that has been demonstrated by specific markers such as vimentin and E-cadherin, respectively (Gerardo-Ramírez et al., 2019). It should be noted that GDF11 has been shown to be a regulator of lipid metabolism, since a decrease in cholesterol levels has been observed in animal models both in cell lines (unpublished data) and in other lipids (Lu et al., 2019). It is important to emphasize that this molecule would not only serve to attack liver tumors, but also, in breast tumors it has shown a reduction in aggressiveness (Bajikar et al., 2017).

Therefore, it is important to study the metabolism of lipids, mainly cholesterol, in the polarization of TAM and the aggressive phenotype offered to transformed cells, which is why the use of inhibitors of the mevalonate pathway or lipid syntheses, such as GDF11 and statins would represent a novel strategy in the treatment of various tumors, including HCC. These strategies are quite specific in HCC, since the liver synthesizes 70-80% of the total cholesterol in the body (J. Yang et al., 2020), in addition to the fact that this organ comprises 80-90% of all macrophages in the human body and cover up to 20% of non-parenchymal cells in the liver (Terai et al., 2017); in the case of tumor tissue, TAM can represent up to 50% of cells, depending on the type of tumor (Vinogradov et al., 2014).

6. JUSTIFICATION

GDF11 tends to carry out the opposite effect to the TGF-ß molecule in the response against liver tumor cells, reducing the aggressive phenotype and modifying cholesterol metabolism, in addition, it has been observed that it may have a relevant role in modulating the microenvironment of these cells, mainly TAM. The study of the activation and acquisition of an antitumor polarity by TAM will allow the opening of new knowledge and strategies for the treatment of various types of tumors including HCC.

7. RESEARCH QUESTION

Does GDF11 treatment affect TAM polarity and its intercommunication with HCC-derived cells?

8. HYPOTHESIS

The treatment with GDF11 will modify the polarization of TAM, reducing the aggressiveness phenotype of HCC derived cells.

9. AIM

To evaluate the polarity of TAM and their role in intercommunication with cells derived from an HCC during GDF11 treatment.

10. SPECIFIC AIMS

- To characterize the signaling pathway mediated by Smad proteins in the macrophage polarity in the presence of GDF11.

- To assess and compare macrophage polarity using conditioned media from HCC-derived lines and anti-inflammatory interleukins.

- To assess the polarity of TAMs in the presence of GDF11.

- To determine the effect of GDF11 on cholesterol metabolism

11. MATERIALS AND METHODS

Human Recombinant Growth Differentiation Factor 11, GDF11 (Peprotech) at 50 ng/mL and Atorvastatin (Sigma) at 10 µM were used following the next experiments. For macrophage differentiation was used PMA 200 ng/mL and LPS 50 ng/mL (Sigma) and the M2 polarization were used IL-4 and IL-13 at 20 ng/mL (Peprotech).

11.1 Cell lines and culture conditions

Huh-7 cell lines were cultured in Williams medium (Sigma-Aldrich, USA), and THP-1 cells were maintained in RPMI 1640 medium (Gibco, 31800-14), both were obtained from ATCC company (Manassas, VA, USA). Medium were supplemented with 10% fetal bovine serum (FBS) (Hyclone, USA) and 1% of antibiotic-antimycotic (Thermo-Fisher, USA). All cells were incubated and maintained at 37 ºC in a humidified 5% CO_2 atmosphere. THP-1 monocytes were treated with PMA (200 ng/mL) at 24 h for macrophage differentiation (M0 macrophages).

11.2 Obtaining conditioned medium (CM) from HCC cell lines

$1X10^6$ Huh-7 cell lines were seeded in 2 mL of culture medium at 37 ºC, 5% CO_2 overnight. Then, the cells were washed with PBS and a Williams culture medium containing 10% FBS was added. After 24 h of incubation, the medium or CM was obtained from the cells derived from HCC and were used 1:1 dilution (working CM) in experimental treatment with THP-1 macrophages. If the medium is not used the moment can be stored at -20 ºC for a couple of weeks.

11.3 Protein Quantification

Protein extraction was performed using lysis buffer containing proteases inhibitors (PhosSTOP, Rocher, Complete, Rocher). Protein quantification was performed using commercial KIT bicinchoninic acid (BSA, Pierce, Thermo Fisher Scientific) following the manufacturer's protocol.

11.4 Western blotting

Western blotting analysis was performed according to standard procedure in cell lines. The cells were seeded in a $1x10^6$ cells confluence and it was used 40µg of protein of each samples, after the proteins were loaded and separated by SDS-PAGE protocol at 120 V for 1 hour and subsequently transferred to a polyvinylidene fluoride membranes (PVDF) during 1.5 hours at 4 ºC and 120V. Non-specific binding was blocked by incubating the membranes with 5% milk for 1 h. The protein-blotted membranes were incubated with different primary antibodies overnight at 4 °C. HRP-conjugated secondary antibodies were incubated at room temperature for 1-2 h. The chemiluminescence signals were developed with Super Signal West Pico Quimioluminiscence (Pierce Biotechnology, USA) and detected using the ChemiDocTM MP Imaging System (BIO-RAD). The protein expression level was quantified and normalized to β-actin as an internal reference with Fiji software (NIH). The information of all antibodies used is listed in **Table 1**.

Table 1. Antibodies list			
Antibody	**Dilution**	**Secondary**	**Cat. Number**
pSmad3 (S423/425)	1:1000 WB	Rabbit mAb	Cell Signaling C25A9
Smad3	1:1000 WB	Rabbit mAb	Cell Signaling C67H9
pSmad2 (S465/467)	1:1000 WB	Rabbit mAb	Cell Signaling 138D4
Smad2	1:1000 WB	Rabbit mAb	Cell Signaling D43B4 XP(R)
Smurf1	1:200 WB	Mouse mAb	Santa Cruz Biotechnology sc-100616
CD206 (15-2)	1:200 WB 0.6 ug FCM	Mouse mAb	Santa Cruz Biotechnology sc-58986
B7-2 (CD86) (BU63)	1:200 WB 0.6 ug FCM	Mouse mAb	Santa Cruz Biotechnology sc-19617
β-catenin	1:1000 WB	Rabbit mAb	Cell Signaling D10A8
β-actin	1:10000 WB	Rabbit	Millipore-Sigma A3854
Alexa Fluor 488	1:500 IF	Mouse mAb	Thermo Fisher A32723

WB: Western blot; FCM: Flow cytometry; IF: Immunofluorescence

11.5 Immunofluorescence assay

For cell immunofluorescence, THP-1 cells were seeded at 70-80% confluence on cover slides treated with collagen type II, and fixed in 4% paraformaldehyde for 20 min, followed by three washes with PBS. Cells were blocked in presence of 5% albumin and

10% of FBS for 30 min and incubated with primary antibodies including CD206 marker (1:100 dilution in 0.5% BSA), followed by secondary antibody staining (1:500 dilution in 0.5% BSA). Nuclei were counterstained with DAPI (1:500 dilution in water) (Thermo Fisher) at 5 min. Photographs were taken using a laser scanning confocal microscope Carl Zeiss.

11.6 Macrophage polarization

For macrophage polarization we used the protocol reported by (Dong et al., 2019) with some modification. Here were seeded $1X10^6$ THP-1 macrophages (M0) and were treated with LPS (100 ng/mL) for 24 h to M1 macrophages formation. Later M0 macrophages were treated in presence of IL-4 (20 ng/mL) and IL-13 (20 ng/mL) in combination at 72 h for M2 macrophages activation. Succesively M0 and M2 macrophages were treated in presence of GDF11 (50ng/mL) each 24 h. These experiments were evaluated using Western Blot and flow cytometry (Figure 5).

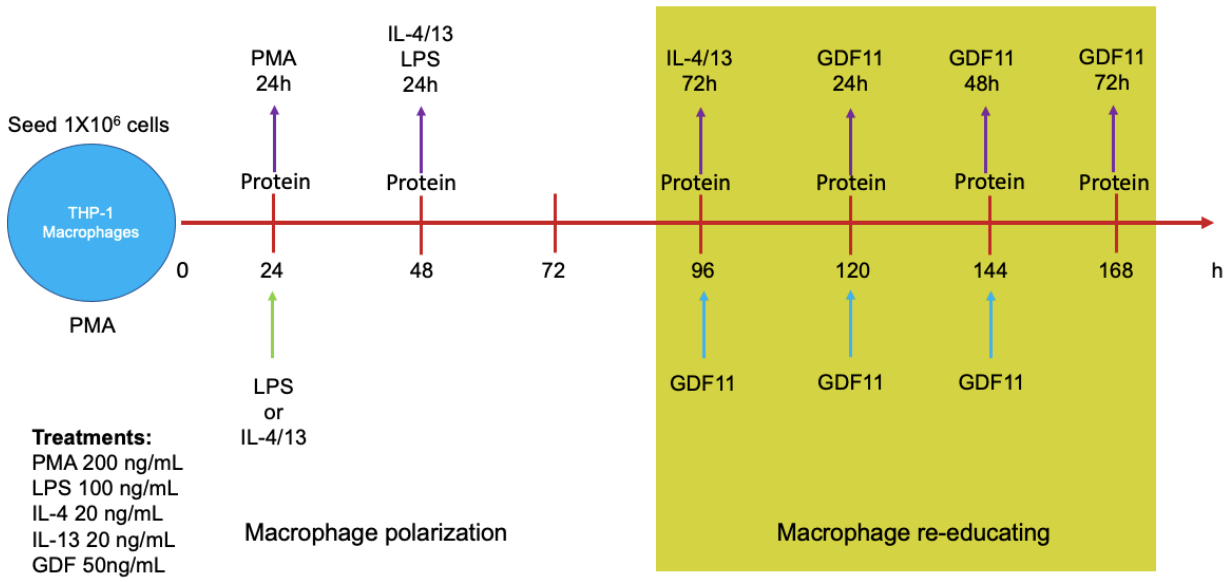


Figure 5. *Macrophage polarization protocol using LPS and IL-4/13 in presence of GDF11.*

11.7 GDF11 re-education protocol

After the M2 polarization process in macrophages, they were treated for another 72 h in the presence of IL-4/13 in combination with GDF11, three doses every 24 h. After treatment, CD206 levels were observed by flow cytometry (Figure 6).

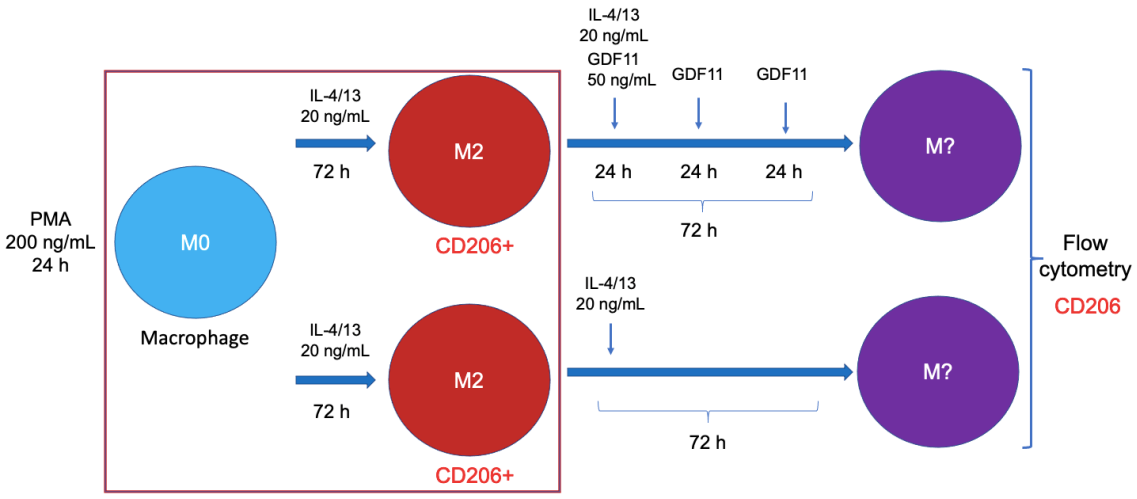


Figure 6. *GDF11 in competence with anti-inflammatory stimulus.*

11.8 Flow cytometry analysis

1×10^6 THP-1-derived macrophages were harvested and washed in PBS, and after different macrophage polarization, cells were detached using PBS-EDTA 5mM at 40 min in ice (Chen et al., 2015). Macrophages were fixed using 4% PFA at 5 min. After, cells were stained using a primary antibody, anti-CD206 and anti-CD86 (0.6 ug per 1×10^6 cells) overnight at 4ºC in 150 rpm. Later, cells were treated using Alexa 488 (1:500 dilution). Antibodies information is shown in Table 1. An isotype-matched IgG from CD86 and CD206 were used as a negative control. Results were analyzed by flow cytometry (FACSCalibur, BD) and different percentage or median fluorescence intensity (MFI) form subsets were calculated using FlowJo X software.

11.9 Cell viability and proliferation assay

Three replications of a total of $1X10^4$ Huh-7 and THP-1 macrophages were seeded per well in 96-well plates overnight and then incubated with GDF11 (50 ng/mL), atorvastatin (5, 10 and 15 µM) for another 24, 48 and 72 h. Once the treatment time was over, 10 µL Cell Counting Kit-8 (CCK-8; Sigma, USA) solution was then added to each well. After incubation at 37 ºC for 1 h, the absorbance at 450 nm was measured on

microplate reader. On the other hand, some cells were fixed using methanol and then stained with 0.5% crystal violet following colorant extraction using 2% SDS solution and absorbance quantification was measured at 622 nm. All experiments were repeated at least three times. AraC (10 µM) and H_2O_2 were used at negative control in proliferation and viability assays, respectively.

11.10 Cholesterol determination

Total cholesterol levels of $1x10^6$ THP-1 derived macrophages were measured as previously reported (Domínguez- Pérez et al., 2019) with some modifications. Briefly, cell lysate was saponified with alcoholic KOH 33% in a 60°C heating block for 15 min, 1ml of hexane and 300µl of distilled water were added and shaken to ensure complete mixing. After evaporation samples were used for cholesterol measurement with ophtaldehyde (OPA) solution dissolved in acetic acid (0.5 mg/ml). Finally, the sulfuric acid was added for 15-30 min and then read at 550 nm in a spectrophotometer.

11.11 Statistical analysis

Data were presented as mean ± standard error of the mean (SEM). The Prism 8 (GraphPad Software, San Diego, CA, USA) software were used for data analysis. The analysis of variance (ANOVA) test was used to compare mean values among three groups whereas the independent-sample two-sided Student's t-test was used to compare two groups with normal distribution data. Statistical significance was indicated by an asterisk (*$P<0.05$).

12. RESULTS

12.1 GDF11 promotes activation of Smad proteins signaling pathway in THP-1 macrophages.

Firstly, to assess the effect of GDF11, THP-1 macrophages (M0) were treated in presence or absence of GDF11 (50 ng/mL) at short times and phosphorylation of Smad2 and Smad3 proteins were observed at 5 minutes (**Figure 7a and 7b**) suggesting a possible activation of this cell type.

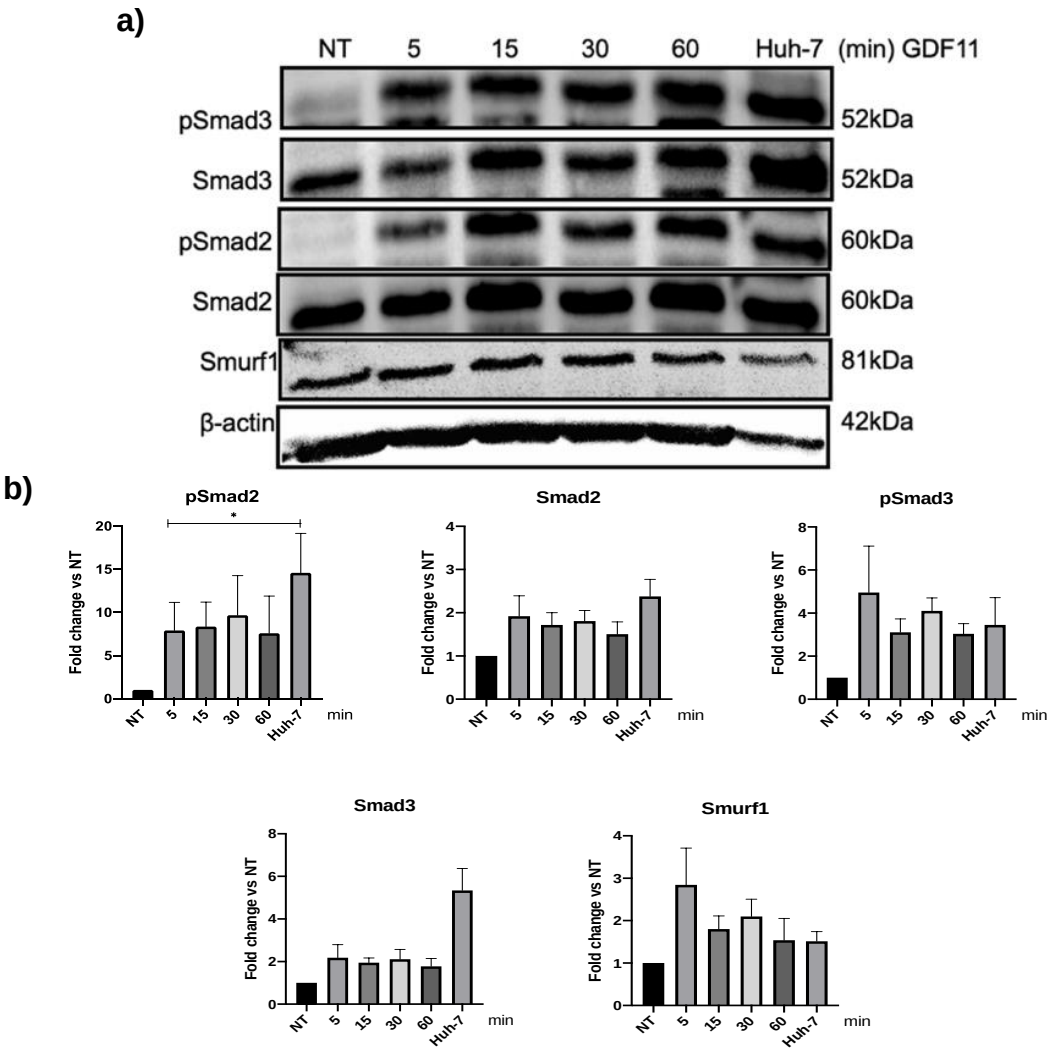
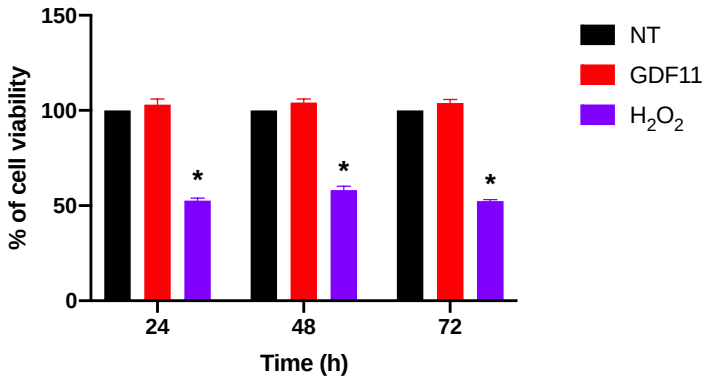


Figure 7. Smad proteins activation in THP-1 macrophages by GDF11. *THP-1 monocytes were diferenciated to macrophages in presence of PMA (200 ng/mL) for 24h. Later macrophages were treated with GDF11 (50 ng/mL) and we observed their activation through Smad proteins phosphorylation by Western blot. Huh-7 cells were used as positive control with GDF11 at 30 minutes. Images a) and densitometric analysis b) are representative of at least three independent experiments. *, p≤0.05 vs NT cells.*

12.2 GDF11 does not affect viability and proliferation in macrophages

Once the activation of the pathway mediated by Smad proteins was observed, inactivated macrophages (M0) were treated in the presence of GDF11 every 24 h until 72 h of treatment, in which it was not observed change in cell viability **(Figure 8a)** or proliferation **(Figure 8b)**.

a)



b)

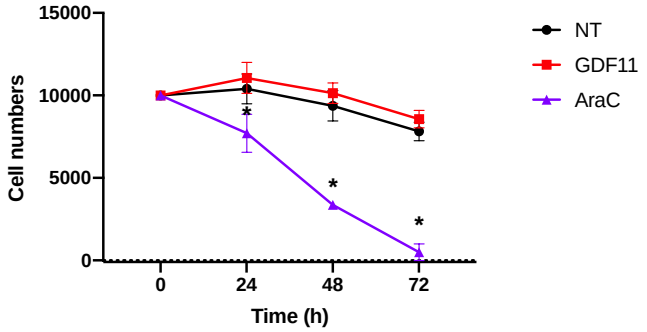
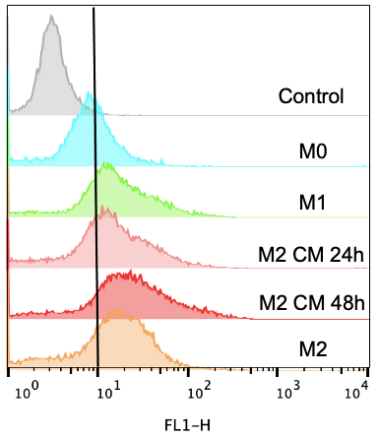


Figure 8. Cell viability and proliferation assay in the presence of GDF11. *Proliferation assay using 0.5% crystal violet in presence or absence of GDF11 (50ng/mL). AraC (10 µM) and H_2O_2 were used as negative controls. Data shown represent 2 independent experiments in triplicate to which analysis of variance (ANOVA) followed by the post hoc Tukey with a significance level * P≤001 vs NT or T0.*

12.3 CD86 marker increase in activated macrophages

An increase in the CD86 marker was observed in the macrophages treated with LPS, after this, the same increase and shift in fluorescence was observed in all treatments **(Figure 9a)**, finding a difference in percentage and the Mean fluorescence Intensity (MFI) of the population compared to inactivated macrophages **(Figure 9b and 9c)**.

a)



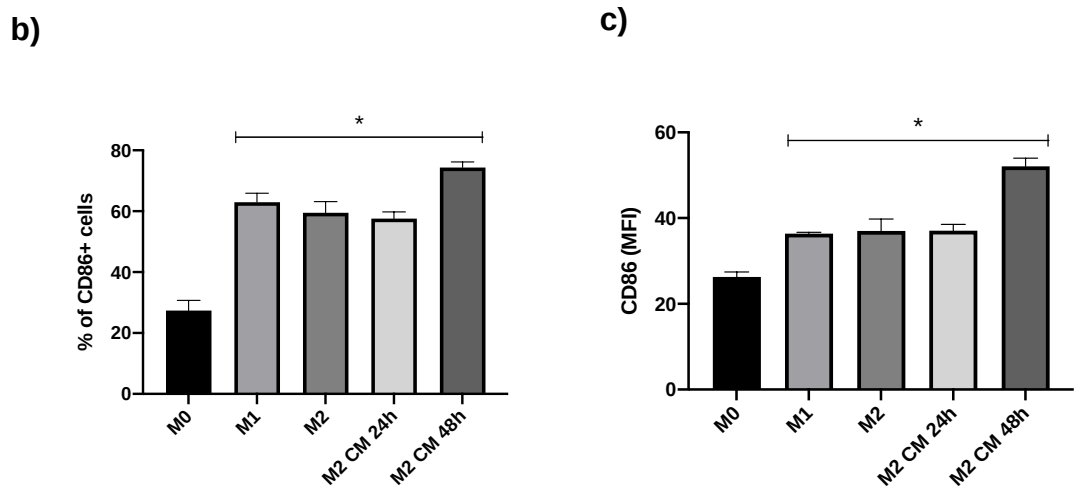


Figure 9. CD86+ in activated macrophages *THP-1 monocytes were diferenciated to macrophages in presence of PMA (200 ng/mL) at 24 h. Macrophages were treated with LPS, anti-inflammatory cytokynes (IL-4/13) or conditioned media (CM) and we observed their activation through CD86 marker by flow cytometry. a**)** Represents the shift in fluorescence, b) bars indicate positive percentage of population and c) bars indicate positive mean fluorescence intensity (MFI). All experiments are representative of at least three independent experiments. *, p≤0.001 vs M0 macrophages.*

12.4 CD206 marker increase in M2 macrophages

An increase in the CD206, M2 macrophage marker, was observed in the macrophages treated with anti-inflammatory cytokines (IL-4/13) or conditioned media (CM) from Huh-7 cells, after this, the same increase and shift in fluorescence was observed in all treatments **(Figure 10a)**, finding a difference in percentage and the MFI of the population compared to inactivated macrophages (M0), M1 and CM 24h treatment **(Figure 10b and 10c)**.

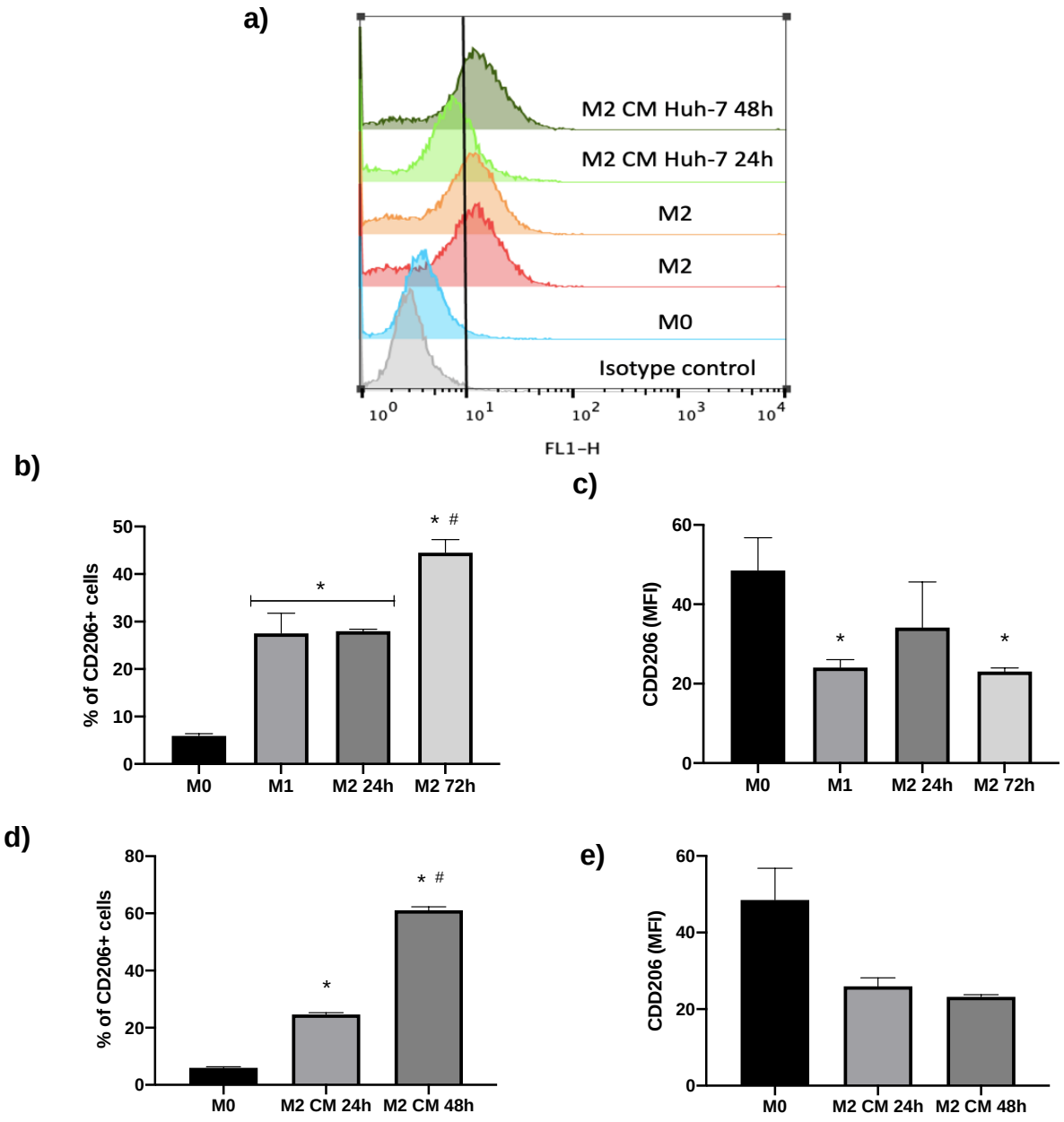


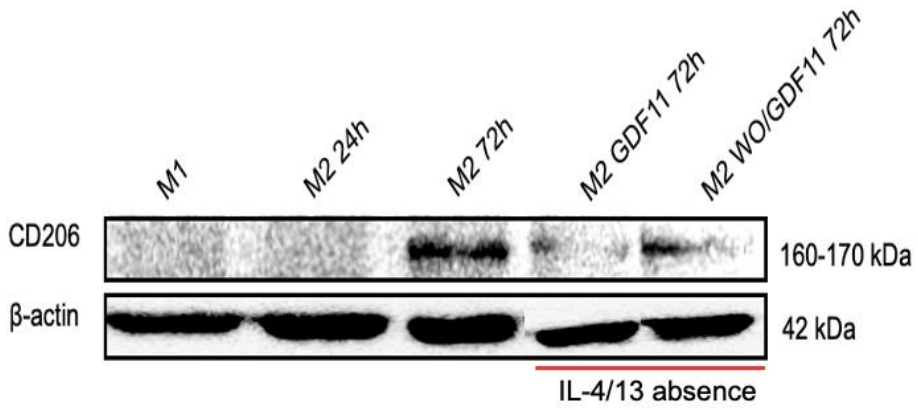
Figure 10. CD206+ in M2 activated macrophages *THP-1 monocytes were diferenciated to macrophages in presence of PMA (200 ng/mL) for 24h. Macrophages were treated with anti-inflammatory cytokynes (IL-4/13) or conditioned media (CM) and we observed their activation through CD206 marker by Flow Cytometry. a**)** Represents the shift in fluorescence of CD206 population, b) and d) bars indicate CD206 positive percentage population, c) and e) bars*

*indicate CD206 positive median fluorescence intensity (MFI). All experiments are representative of at least three independent experiments. *, p≤0.001 vs M0 macrophages, #, p≤0.001 vs M1, M2 24h and M2 CM 24h macrophages.*

12.5 The absence of interleukins decreases the content of CD206

To test the effect of GDF11 on M2 macrophages, total protein content in activated macrophages with different polarity was analyzed, where it was observed that the absence of anti-inflammatory interleukins (IL-4/13) was responsible for reducing the CD206 marker and not due to the effect of GDF11 **(Figure 11a)**. M2 macrophages lose their polarity after 72 h in the absence of interleukins.

a)



The inactivated macrophages have a high protein content of CD206 which is decreased by the effect of GDF11 **(Figure 11b)**, indicating that it could be involved in the loss of polarity in M2 macrophages.

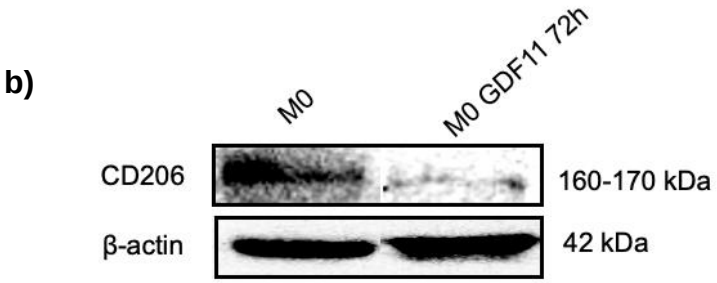
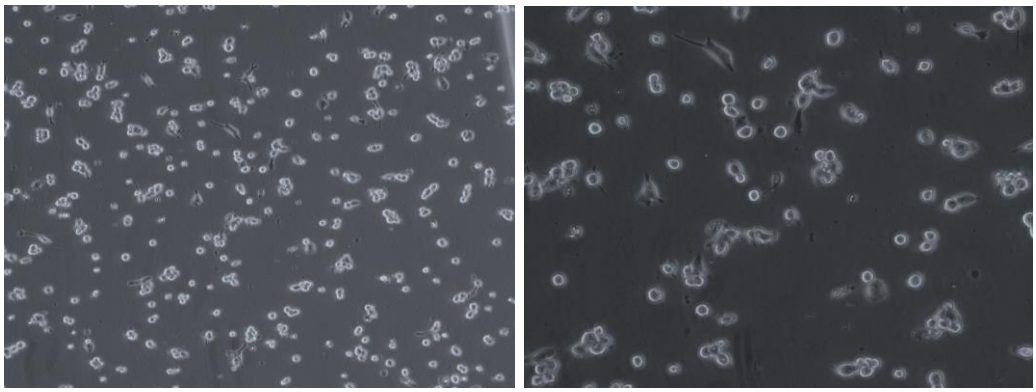


Figure 11. The absence of interleukins decreases the content of CD206. *THP-1 monocytes were diferenciated to macrophages in presence of PMA (200 ng/mL) for 24h. Later macrophages were treated with LPS, anti-inflammatory cytokynes (IL-4/13), GDF11 (50 ng/mL) or in GDF11 absense (WO, without GDF11) and we observed their activation through CD206 marker by Western blot. a**)** Show content of CD206 in macrophages and b) Is observed the effect of GDF11 in M0 macrophages.*

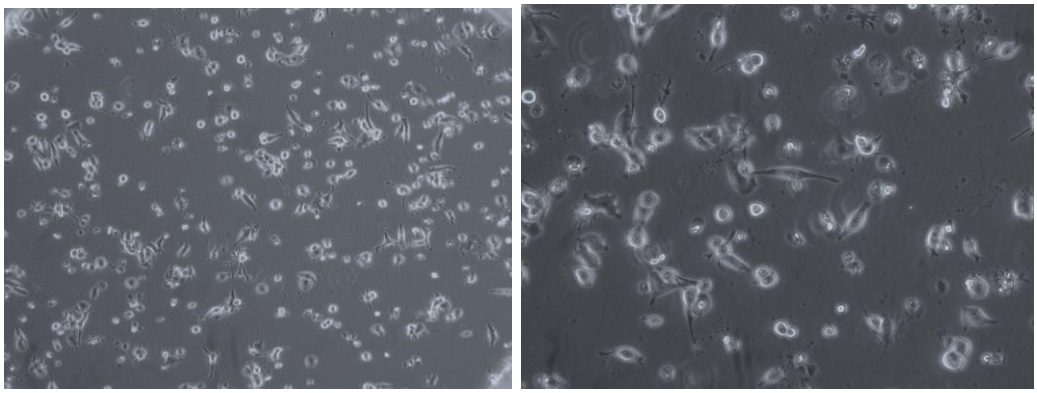
12.6 Morphology of polarizing macrophages

To observe the effect mediated by the different molecules that promote macrophage activation, morphologies of differentiated macrophages were analyzed, observing that macrophages with M2 polarity acquire a more extended or fibroblast-like morphology **(Figure 12)**.

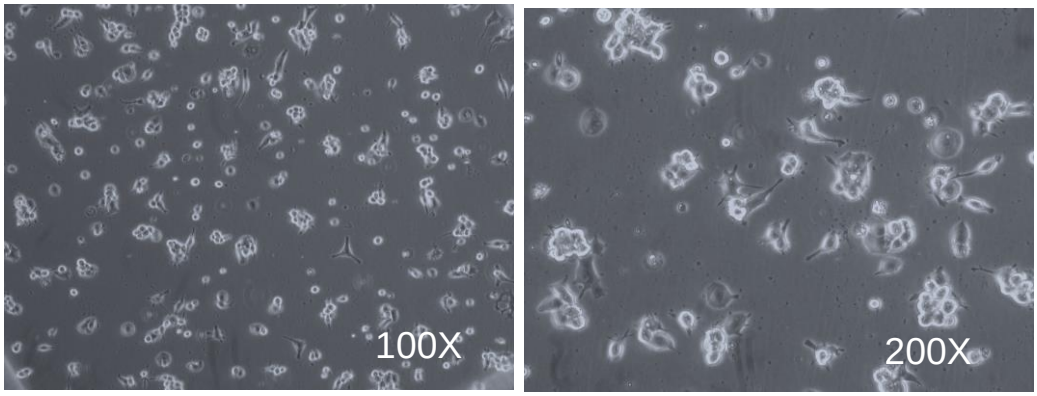
M0



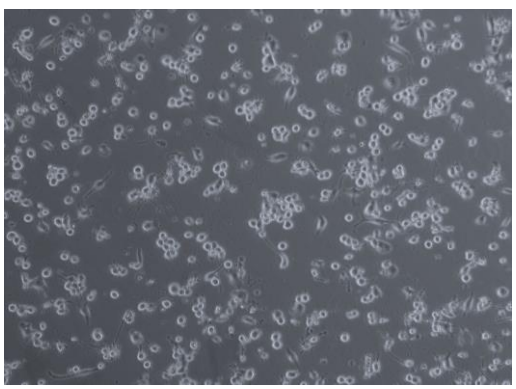
M1

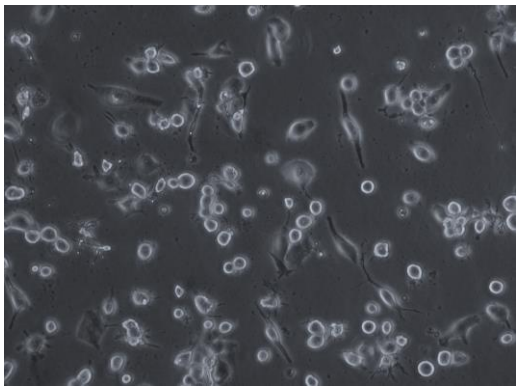


M2 24 h

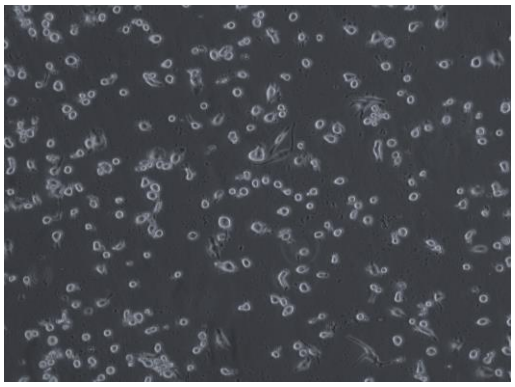


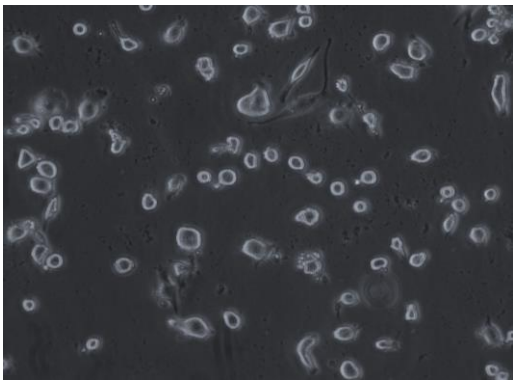
M2 72 h



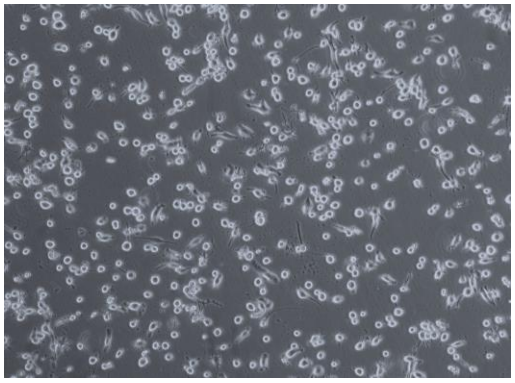


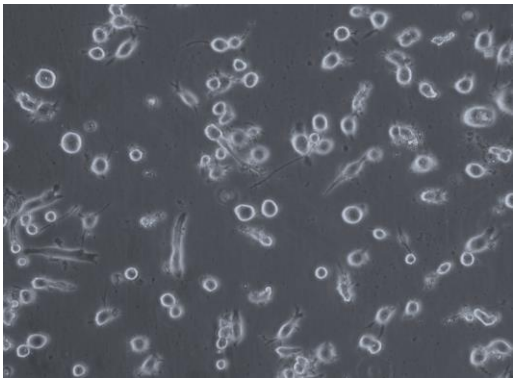
M2
GDF11
72 h



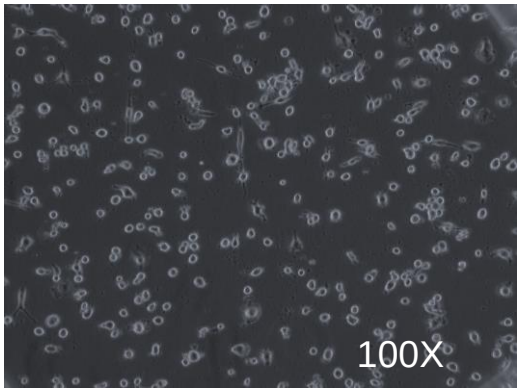


M0
GDF11
72 h





M2
without
GDF11
72h



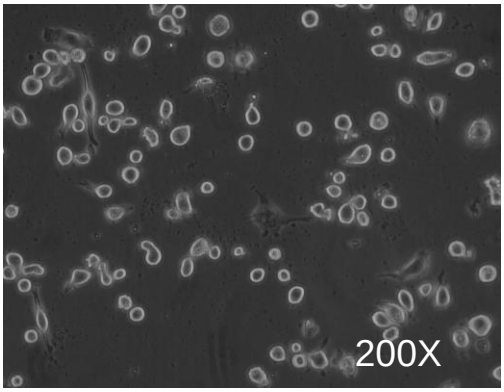


Figure 12. Morphologies of polarized macrophages. *Morphologies of activated macrophages were observed using brightfield microcopy, data is shown at 100X and 200X. Images are representative of at least three independent experiments.*

12.7 GDF11 increased CD206 marker in M0 macrophages

To corroborate the effect of GDF11 by flow cytometry, inactivated macrophages (M0) were treated in the presence of GDF11, in which an increase in the CD206 marker was observed, suggesting that they are acquiring an M2 polarity, this effect is notably observed at 48 and 72 h **(Figure 13)**.

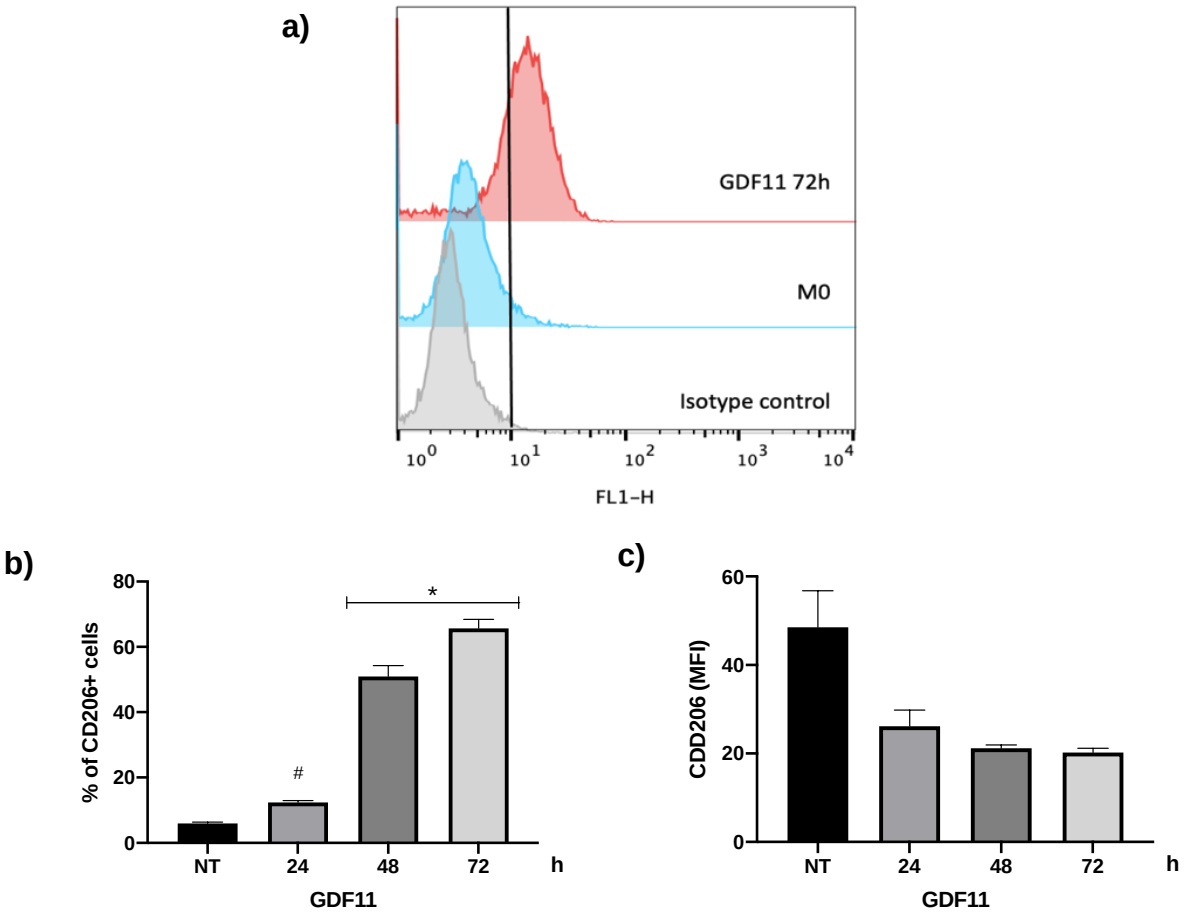


Figure 13. CD206+ in M2 activated macrophages *THP-1 monocytes were diferenciated to macrophages in presence of PMA (200 ng/mL) for 24h. Later macrophages were treated with GDF11 (50 ng/mL) at 72 h. CD206 marker was detected by Flow Cytometry. a**)** Represents the shift in fluorescence of CD206 population, b) bars indicate CD206 positive percentage population and c) bars indicate CD206 positive median fluorescence intensity (MFI). All experiments are representative of at least three independent experiments. *, p≤0.0001 vs NT macrophages, #, p≤0.001 vs NT macrophages.*

12.8 GDF11 in competence with IL-4/13 decrease CD206 marker in M2 macrophages

An increase in CD206 was again observed due to treatment with IL-4/13 for 72 h. To avoid the loss in polarity in these macrophages, an additional treatment with interleukins was applied in the presence of GDF11. Treatment for an additional 72 h (M2 post-treatment) with interleukins increases the M2 macrophage population by up to 90% compared to 44% for just one application **(Figure 14a and 14b)**. Additional treatment in the presence of GDF11 decreases the M2 macrophage population, having a behavior similar to that of macrophages treated with a single dose of interleukins.

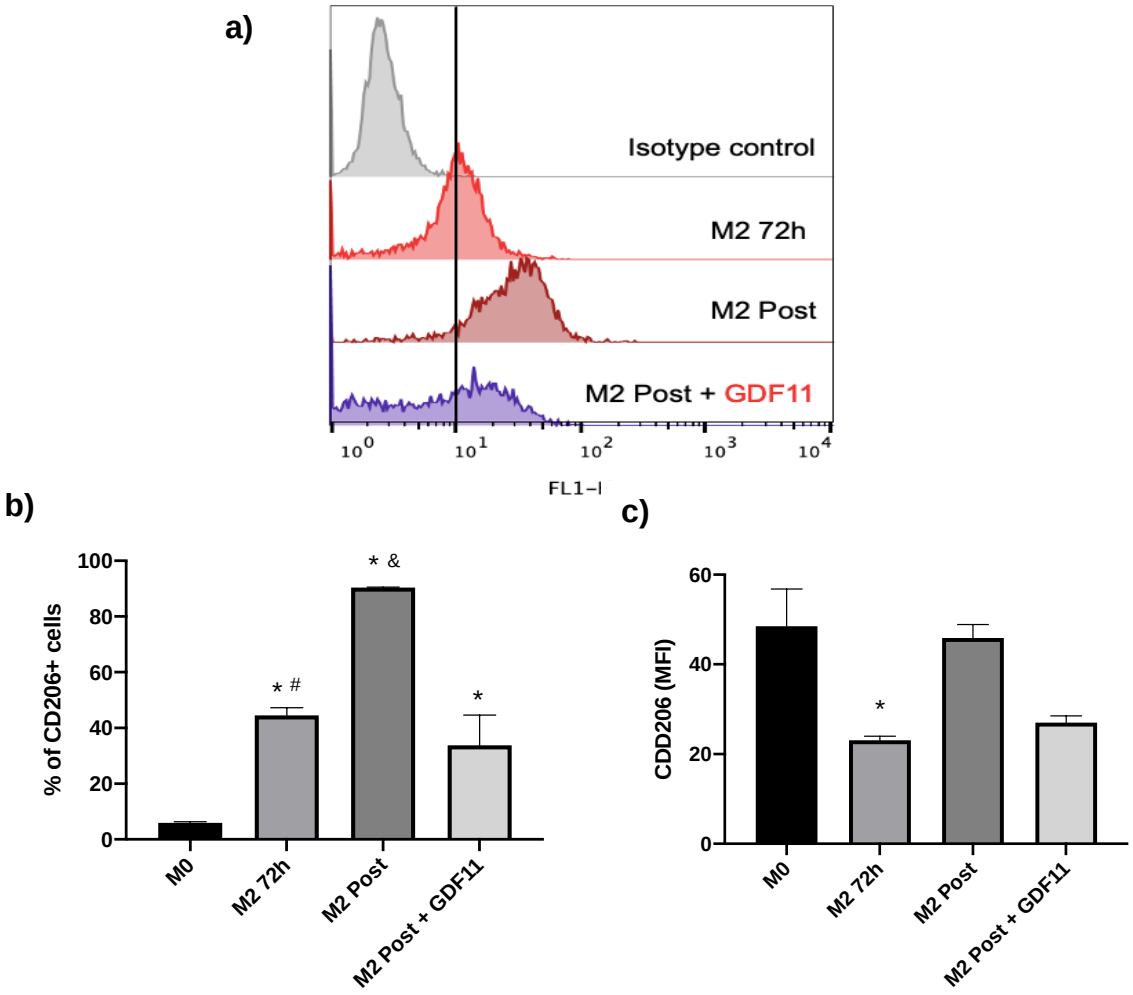


Figure 14. CD206+ in M2 activated macrophages *THP-1 monocytes were diferenciated to macrophages in presence of PMA (200 ng/mL) for 24h. Late the macrophages were treated with anti-inflammatory cytokynes (IL-4/13) for 72 h and postreatment with the same concentration (M2 Post). GDF11 (50 ng/mL) was aplied in macrophages in presence of interlekines for 72 h postreatment. CD206 marker was detected by Flow Cytometry. A**)** Represents the shift in fluorescence of CD206 population, B) bars indicate CD206 positive percentage population and C) bars indicate CD206 positive median fluorescence intensity (MFI). All experiments are representative of at least three independent experiments. *, p≤0.001*

vs M0 macrophages, #, p≤0.001 vs M2 Post and, &, p≤0.001 vs M2 Post + GDF11 macrophages.

12.9 GDF11 in competence with IL-4/13 do not affect macrophage morphologies

After observed the decrease of M2 macrophage marker in competence with anti-inflammatory cytokines and GDF11 treatment, it was analyzed the macrophage morphologies finding that there was not any change notwithstanding the decreased of CD206 **(Figure 15)**.

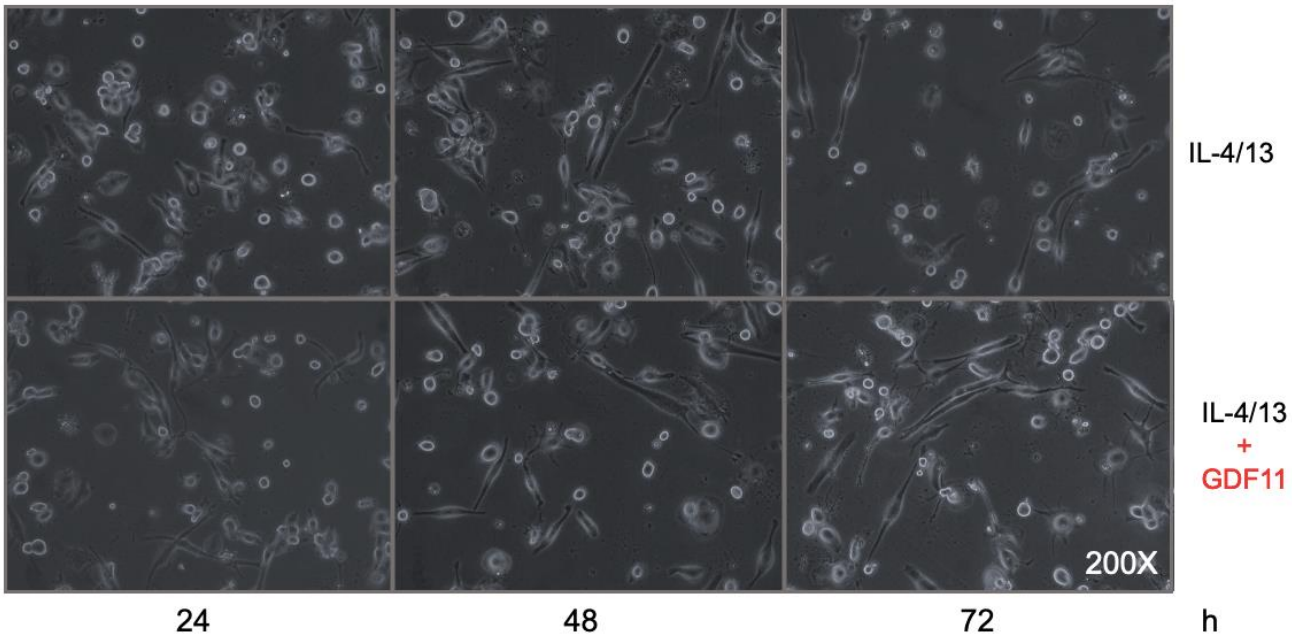


Figure 15. Macrophage morphologies in competence between IL-4/13 and GDF11. *The morphologies of M2 activated macrophages observed by brightfield microcopy is shown at*

200X. Macrophage acquire extended and bipolar structures. Images are representative of at least three independent experiments.

12.10 Conditioned media from HCC cells increased CD206 marker in macrophages

Conditioned media from Huh-7 cells increased the CD206 in THP-1 macrophages in presence or absence of GDF11 at 48 h, suggesting an alternative macrophage activation. It was observed an increase of CD206 in the macrophage's cytoplasm treated with the CM **(Figure 16)**.

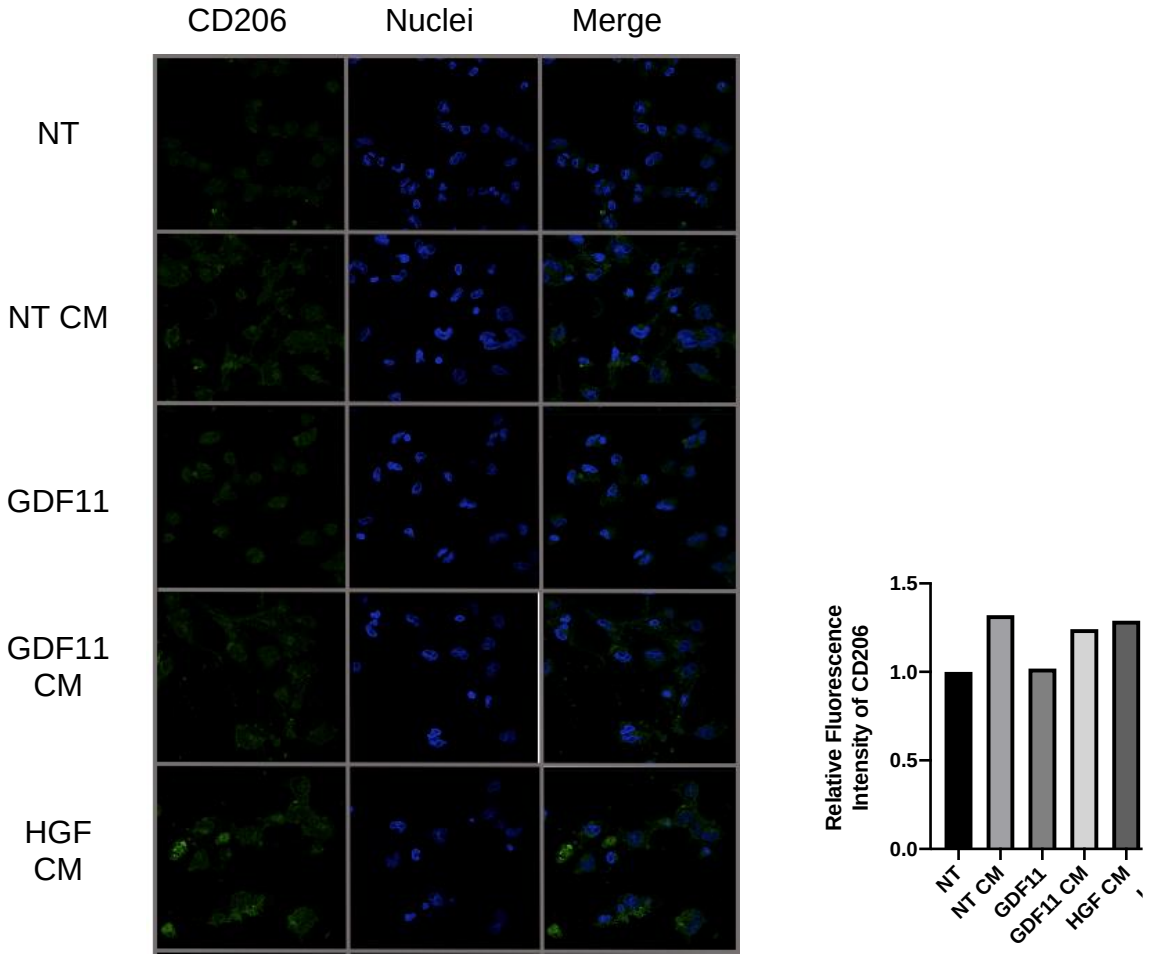


Figure 16. CD206 marker in macrophages treated with CM. *THP-1 cells were treated with CM from Huh-7 cells treated or not with GDF11 (50 ng/mL) and fluorescence was observed by confocal microscopy (Carl Zeiss), 400X (a), CD206 (green) and DAPI (Blue). Relative Fluorescence Intensity of CD206 was represent in Graph (b). Was used HGF (50 ng/mL) as control.*

12.11 GDF11 decreased cholesterol levels in THP-1-derived macrophages

To observe if GDF11 has the same metabolic effects in lowering total cholesterol in this cell line, inactivated macrophages were treated with GDF11 every 24 h, thus fulfilling the 72 h of exposure. In the same way as Huh-7 or Hep3B cells (unpublished data), cholesterol levels were decreased in this cell line in a time of 72 h **(Figure 17a)**. No change in cell morphology was observed **(Figure 17b)**.

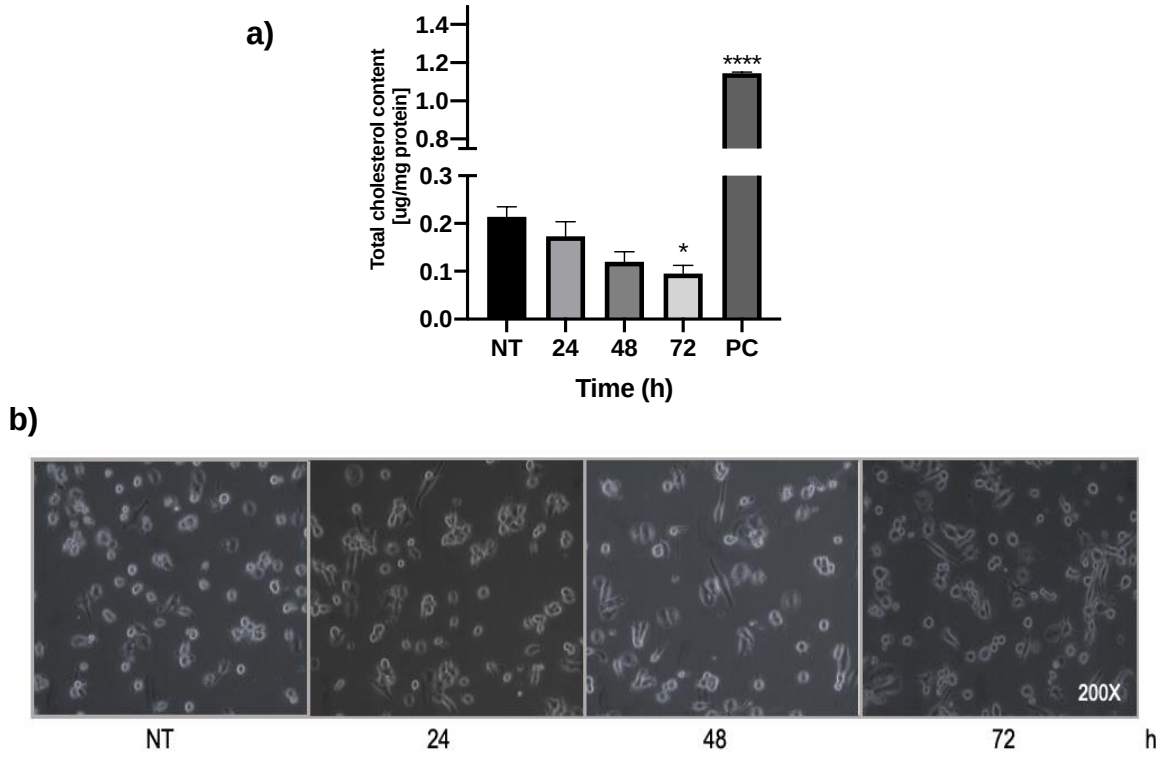


Figure 17. *Cholesterol determination in THP-1 derived macrophages. Macrophages were treated in presence of GDF11 (50 ng/mL) at 72h, and cholesterol levels were quantified using OPA reaction, (figure 17a). The data shown represent three independent experiments in triplicate to which analysis of variance (ANOVA) followed by the post hoc Tukey with a significance level * P≤0.05 vs NT cells. **** P≤0.001 vs NT cells. Tissue from normal liver was as positive control (PC). Images are representative of at least three independent experiments and observed by brightfield microcopy is shown at 200X.*

12.12 Atorvastatin decreased cholesterol levels in THP-1-derived macrophages

Previous experiment confirmed that GDF11 decreases intra-cellular cholesterol content, for this reason a similar test was carried out using statins. This was confirmed with atorvastatin (10 µM) that also decreases the content of this lipid, showing that both molecules work similarly (Figure 18a). It is also observed that cell viability decreased at 72 h of treatment **(Figure 18b)**. Also, we identify changes in cell morphology in the last treatment at 72 h **(Figure 18c)**.

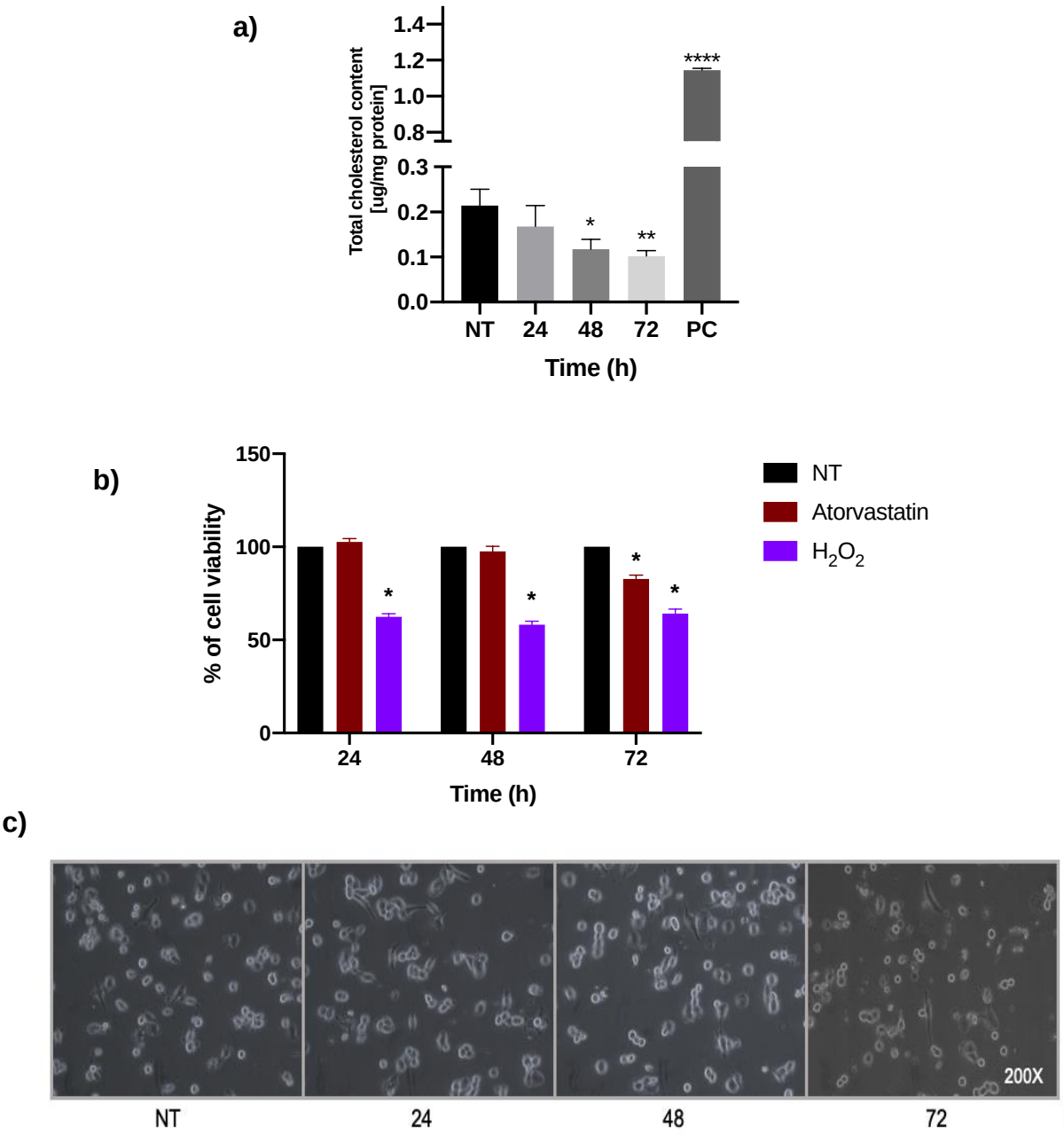


Figure 18. *Cholesterol determination in THP-1 derived macrophages. Macrophages were treated in presence of Atorvastatin (10 µM) at 72h, and cholesterol levels were quantified using OPA, figure 18 a. The data shown represent three independent experiments in triplicate to which analysis of variance (ANOVA) followed by the post hoc Tukey with a significance level Figure 18b. Tissue from normal liver was as positive control (PC). Cell viability evaluated using*

*crystal violet 0.5% were used un Figure 18b. Images are representative of at least three independent experiments and observed by brightfield microcopy is shown at 200X. P≤0.05 vs NT cells, **** P≤0.001 vs NT cells.*

12.13 Atorvastatin affects cell viability and cell number in THP-1-derived macrophages

To corroborate cell viability, macrophages were treated in the presence of different atorvastatin concentrations (5, 10 and 15 µM) until reaching 72 h of treatment. Observing that longer time, cell viability and number of cells decrease remarkably **(Figure 19a and 19b)**.

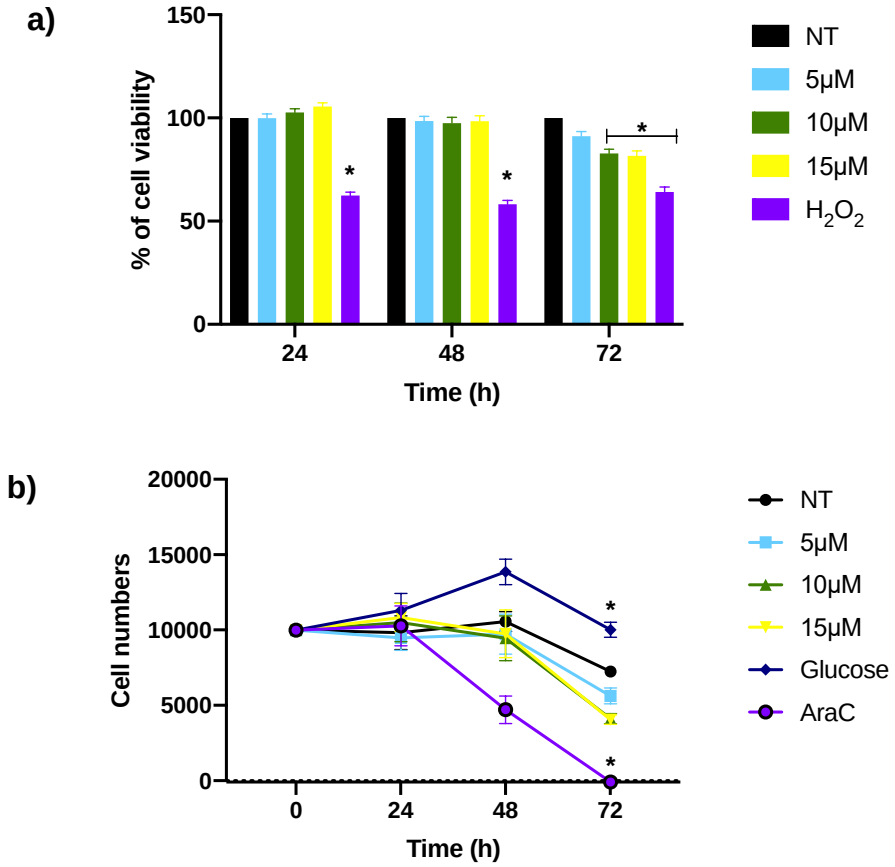
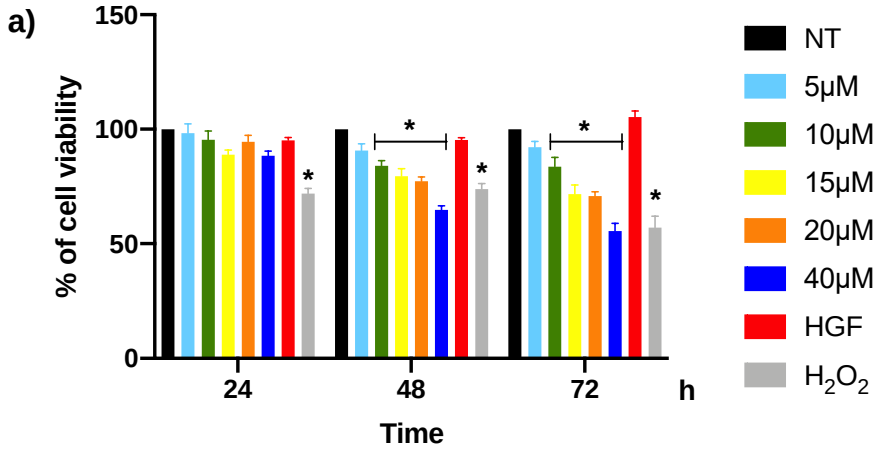


Figure 19. Proliferation and cell viability assay in the presence of atorvastatin. *THP-1 derived macrophages were treated for 72 h at different concentrations of atorvastatin (5, 10 and 15 µM). Glucose was used as a positive control; AraC (10 µM) and H_2O_2 as negative controls. The data shown represent two independent experiments in triplicate to which analysis of variance (ANOVA) followed by the post hoc Tukey with a significance level * P≤0.05 vs NT or NT T0.*

12.14 Atorvastatin decreased cell viability and cell number in Huh-7 cell line.

Finally, to corroborate cell viability and proliferation in HCC cells lines and compare the effect of statins respect to macrophages, Huh-7 cells were treated in the presence of different atorvastatin concentrations (5, 10, 15, 20 and 40 µM) until reaching 72 h treatment. Observing at longer time, the cells viability decrease remarkably but number cells increased in time. Interestingly, concentration of 10 µM used in macrophages also affects this cell type, inhibiting or reducing number cells compared non-treated cells **(Figure 19a and 19b)**.



b)

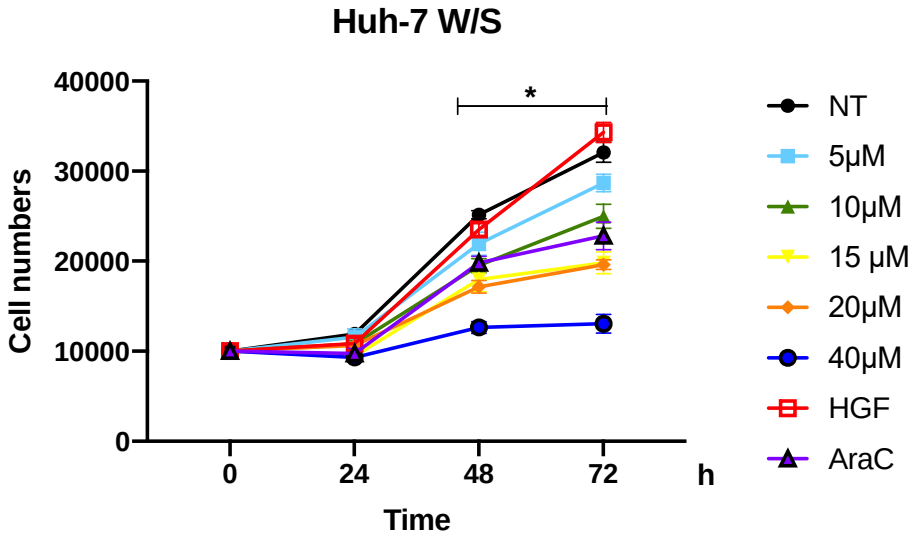


Figure 20. Proliferation and cell viability assay in the presence of atorvastatin. *Huh-7 cells were treated for 72 h at different concentrations of atorvastatin (5, 10, 15, 20 and 40 μM). HGF (50 ng/mL) was used as a positive control; AraC (10 μM) and H_2O_2 as negative controls. The data shown represent two independent experiments in triplicate to which analysis of variance (ANOVA) followed by the post hoc Tukey with a significance level * P≤0.05 vs NT or NT T0.*

13. DISCUSSION

In this work, it was shown that GDF11 ligand is involved in the activation of signaling pathways that involve Smad proteins that indirectly promote macrophage polarization depending on the activation state, in addition to decreasing in the metabolic context total cholesterol levels. This decrease in cholesterol was corroborated through the use of statins, finding a similar effect, with this we can suggest that GDF11 acts indirectly and in the opposite way to TGF-β.

As has been recently reported in our research group, GDF11 ligand promotes the loss of the aggressive phenotype in HCC-derived cell lines such as Huh-7 and Hep3B. Studied by key experiments, decreased proliferation and increased mesenchymal-epithelial transition were reported. Binding of GDF11 with its receptor promotes activation of Smad proteins, mainly Smad2 and Smad3 through specific phosphorylation, which leads to signal transduction (Gerardo-Ramírez et al., 2019).

In this work we demonstrate that in macrophages differentiated from the THP-1 monocyte cell line, GDF11 (50 ng/mL) activated the signaling pathway mediated by Smad proteins in approximately 5 min. In the same way that in the transformed hepatocytes transformed. It was confirmed that GDF11 does not alter cell viability or proliferation, and do not have a cytotoxic effect in this cell line.

Macrophages are very versatile cells that respond quickly to different conditions that arise in the environment and depending on them develop a specific polarity, in such a way that we would expect to see a change in their polarity under the effect of GDF11.

Its counterpart, TGF-β, also secreted by TME, promotes alternative activation in macrophages (Gong et al., 2012). M2 activation in macrophages is due to a very important transcription factor known as SNAIL, which is also present and in high concentration in tumor cells (Zhang et al., 2016b). SNAIL inhibition promotes a change in polarity, favoring M1 polarity, which would indicate an anti-tumor response(Bose et al., 2019).

Under these described effects, it was proposed to standardize the polarity of macrophages using the treatments described in the methodology, where it was found that the CD86 marker is present in macrophages with different treatments, including those treated with LPS. On the contrary, the anti-inflammatory interleukins IL-4/13 and the conditioned medium (CM) derived from cancer cell lines differentially increased the CD206 positive population showing a greater increase in macrophages treated with CM (60%), compared to those treated with interleukins (40%). Which suggests that in CM there are molecules capable of differentiating from macrophage, for example, WNT ligands as reported in Hepa 1-6 cells (Y. Yang et al., 2018). These polarities were corroborated by bright field microscopy, observing a fibroblast, bipolar or extended morphology in macrophages with alternative polarity (Dong et al., 2019).

In polarized macrophages, it was observed that GDF11 has a differential effect that depends on its activation state. Inactivated macrophages treated for 72 h with GDF11 increased the percentage of CD206 positive cells, indicating a possible alternative activation of this cell line, as would its homologue TGFβ (Zhang et al., 2016a).

It was observed that in the macrophages polarized to M2 treated with GDF11, the protein content of CD206 decreased, suggesting the loss of polarity, however, this

result was the result of the absence of IL-4/13. On the other hand, inactivated macrophages have a high content of CD206, however, treatment with GDF11 reduces the concentration of this marker. Taking into account that CD206 is an M2 polarity marker, it was observed by flow cytometry that GDF11 increases the percentage of M2 macrophages but tends to decrease the MFI corresponding to CD206.

After this loss of M2 polarity, in a competition test between IL-4/13 and GDF11 for an additional 72 h of treatment, it was observed that GDF11 does decrease the population of macrophages positive for CD206 compared to its control, only interleukins, demonstrating the effect of GDF11 on alternately polarized macrophages. Although, question remains to be clarified if GDF11 decreases or only prevents the increase of this macrophage subsets, since the increase in the exposure time of interleukins increased the positive population for CD206 by up to 90%, while the combination kept it below a 40%. No changes were found in fibroblast morphologies, but it would be important to determine later a cellular response using these experimental models.

The loss of the CD206 marker has been reported to be involved in the re-polarization of macrophages towards an anti-tumor phenotype. On the other hand, it is important to mention that the activation of this receptor also promotes this process, but also involves the death by apoptosis of most M2 macrophages and it has been seen that the decrease in the M2 population favors survival in patients and also in mice (Jaynes et al., 2020).

GDF11 has been found involved in metabolic issues such as the reduction of lipid levels, mainly cholesterol in serum of murine models fed high-lipid diets (Lu et al., 2019,

p. 11). In our research group, GDF11 has been shown to decrease total cholesterol levels in HCC, Huh-7 and Hep3B cell lines within 72 h (unpublished data). Cholesterol provides an aggressive phenotype to different types of tumors, including HCC (Enríquez-Cortina et al., 2017). In addition, tumor cells can benefit from the cholesterol of cells in the microenvironment, mainly macrophages, promoting its synthesis and release due to the conditions exerted by TME (Goossens et al., 2019).

GDF11 at 72 h of treatment, decreases total cholesterol levels in macrophages, which would indicate that these macrophages are preventing an M2 macrophage response (van Tits et al., 2011), a hypothesis that contradicts the increase in CD206.

With these results, we are seeing a change in cholesterol metabolism, which we corroborate through the use of statins, a dose of atorvastatin was sufficient to lower total cholesterol levels at 48 and 72 h of treatment. With this data we can hypothesize that GDF11 could be indirectly inhibiting the mevalonate pathway, having the opposite effect to TGFβ that promotes cholesterol synthesis by increasing the enzyme HMG-CoA R (Yamane et al., 2016).

In a confirmatory way, the effect of atorvastatin in Huh-7 cells was evaluated and it was observed that the higher the concentration decreases cell viability and proliferation. The same concentration used in macrophages, 10uM in a single dose, was sufficient to delay this effect, confirming its function in both cell types.

With these data we can hypothesize that GDF11 and the use of statins in combination could promote the loss of M2 polarity by TAMs and decrease the aggressive phenotype of the tumor cell.

14. CONCLUSIONS

- THP-1 derived macrophage cell lines respond to GDF11 ligand observed in the activation of the Smad2/3 proteins by specific phosphorylations. In addition, a cytotoxic effect was not observed in cells evaluated in viability and cell proliferation assays.
- A good standardization of macrophage polarities was carried out in M1 and M2 macrophages. In addition, a more efficient M2 polarity was observed through the use of Huh-7 cell conditioned media.
- The molecule GDF11 promotes M2 polarization in macrophages observed by the increase of the CD206 marker. Opposite effect was evaluated when a cotreatment is exerted through the use of IL-4/13 and where a decrease in CD206 levels was observed.
- GDF11 promotes a decrease in total cholesterol content in macrophages, the same effect was observed in treatment with atorvastatin, indicating that both molecules work the same in this type of leukocyte and in HCC-derived cells.
- GDF11 ligand is postulated as a molecule that can directly attack the tumor and the cells of the tumor microenvironment such as macrophages by involving their polarity.

14.1 General scheme of GDF11 in HCC tumors

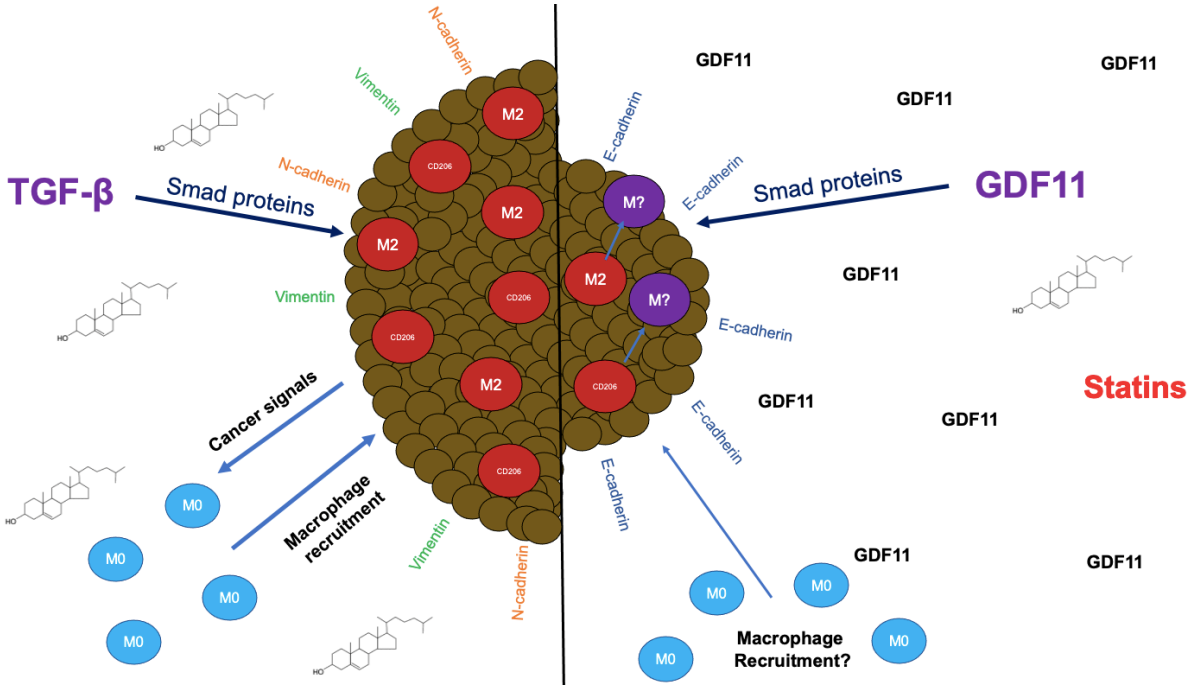


Figure 21. *GDF11 decreased aggressiveness phenotype in HCC tumors, similar effect in their TME, specifically in M2-TAM.*

15. PERSPECTIVES

In order to demonstrate the effect that polarized macrophages with GDF11 have against HCC-derived cells, cell migration models will be worked using migration chambers and determining whether these cells lose their aggressive phenotype by observing the displacement of the cells. After this, animal models in mice will be designed through the generation of tumors by orthotopic models and the effect of macrophages with different polarities on tumor growth will be observed.

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ACTA DE EXAMEN DE GRADO

No. 00218
Matrícula: 2183801892

THE ROLE OF GROWTH DIFFERENTIATION FACTOR 11 ON MACROPHAGE POLARIZATION AND ITS IMPLICATIONS IN HEPATOCELLULAR CARCINOMA.

Con base en la Legislación de la Universidad Autonóma Metropolitana, en la Ciudad de México se presentaron a las 10:00 horas del día 26 del mes de noviembre del año 2020 POR VÍA REMOTA ELECTRÓNICA, los suscritos miembros del jurado designado por la comisión del Posgrado:

DRA. ROXANA URI MIRANDA LABRA
DR. JULIO CESAR ALMANZA PEREZ
DRA. MARIA GUADALUPE ISABEL DOMINGUEZ GOMEZ
DRA. MARIA DEL REFUGIO CLAVIJO CORNEJO

OSCAR ALEJANDRO ESCOBEDO CALVARIO

ALUMNO

Bajo la Presidencia de la primera y con carácter de Secretaria la última, se reunieron para proceder al Examen de Grado cuya denominación aparece al margen, para la obtención del grado de:

MAESTRO EN BIOLOGIA EXPERIMENTAL

DE: OSCAR ALEJANDRO ESCOBEDO CALVARIO

y de acuerdo con el artículo 78 fracción III del Reglamento de Estudios Superiores de la Universidad Autónoma Metropolitana, los miembros del jurado resolvieron:

APROBAR

Acto continuo, la presidenta del jurado comunicó al interesado el resultado de la evaluación y, en caso aprobatorio, le fue tomada la protesta.

REVISÓ

MTRA. ROSALIA SERRANO DE LA PAZ
DIRECTORA DE SISTEMAS ESCOLARES

DIRECTORA DE LA DIVISIÓN DE CBS

DRA. SARA LUCIA CAMARGO RICALDE

PRESIDENTA

DRA. ROXANA URI MIRANDA LABRA

VOCAL

DR. JULIO CESAR ALMANZA PEREZ

VOCAL

DRA. MARIA GUADALUPE ISABEL DOMINGUEZ GOMEZ

SECRETARIA

DRA. MARIA DEL REFUGIO CLAVIJO CORNEJO