



UNIVERSIDAD AUTÓNOMA METROPOLITANA UNIDAD IZTAPALAPA

División de Ciencias Biológicas y de la Salud

Posgrado en Biología Experimental

GDF11 mitigates the pro-tumoral response of M2-like macrophages on HCC cells.

TESIS:

Que para obtener el grado de

Doctor en Biología Experimental

PRESENTA:

Mtro. Biol. Exp. Oscar Alejandro Escobedo Calvario

Matrícula: 2183801892 ORCID: 0000-0002-8978-3122

Correo electrónico: escobedoe17@gmail.com

COMITÉ TUTORAL:

Directora: Dra. María Concepción Gutiérrez Ruiz.

Asesor: Dr. Rogelio Hernández Pando.

Asesor: Prof. Dr. med. Jens Uwe Marquardt.

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Iztapalapa, Ciudad de México, a 19 de noviembre de 2025.



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GDF11 mitigates the pro-tumoral response of M2-like macrophages on HCC cells.

THESIS:

To obtain a

Ph.D. degree in Experimental Biology

PRESENTED BY:

Oscar Alejandro Escobedo Calvario, MSc.

Number: 2183801892

ORCID: 0000-0002-8978-3122

Email: escobedoae17@gmail.com

COMMITTEE:

Director: María Concepción Gutiérrez Ruiz, Ph.D.

Advisor: Rogelio Hernández Pando, Ph.D.

Advisor: Prof. Dr. med. Jens Uwe Marquardt.

DEGREE EXAMINATION JURY:

President: Cédric Coulouarn, Ph.D.

Secretary: Pablo Gustavo Damián Matzumura, Ph.D.

Vocal. Javier Esteban Jiménez Salazar, Ph.D.

Vocal. Benjamín Pérez Aguilar, Ph.D.

Iztapalapa, Mexico City, November 19th, 2025.

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Este trabajo de investigación doctoral fue realizado en el “Laboratorio de Carcinogénesis y Medicina Experimental” en el área de Medicina Experimental y Traslacional del departamento de Ciencias de la Salud de la Universidad Autónoma Metropolitana, Unidad Iztapalapa (UAM-I).

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María Concepción Gutiérrez Ruiz, Ph.D.

Investigador Nacional Emérito.

Laboratorio de Fisiología Celular y Medicina Translacional. Área de Medicina Experimental y Traslacional, Departamento de Ciencias de la Salud. Universidad Autónoma Metropolitana Unidad Iztapalapa (UAM-I), Ciudad de México, México.

Email: mcgr@xanum.uam.mx Work phone: (55) 58044730.

ADVISOR / ASESOR:

Rogelio Hernández Pando, Ph.D.

Investigador Nacional Nivel III

Departamento de Patología Experimental, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (INCMNSZ). Ciudad de México, México.

Email: rogelio.hernandez@incmnsz.mx Work phone (55) 17584848

ADVISOR / ASESOR:

Prof. Dr. Jens Uwe Marquardt.

Medical Clinic I of the University Medical Center Schleswig-Holstein (UKSH), Lübeck Campus, Lübeck, Germany.

Email: Jens.Marquardt@uksh.de Work phone: +49 (0)451 500 44101

El jurado designado por la Comisión Académica del Posgrado en Biología Experimental de la División de Ciencias Biológicas y de la Salud de la Universidad Autónoma Metropolitana aprobó la tesis titulada: “**GDF11 mitigates the pro-tumoral response of M2-like macrophages on HCC cells**” que presentó **Oscar Alejandro Escobedo Calvario**.

SYNODALS / SINODALES

PRESIDENT / PRESIDENTE:

Dr. Cédric Coulouarn

INSERM, Université de Rennes, Rennes, France.

Email: cedric.coulouarn@univ-rennes.fr Work phone +33223233881

SECRETARY / SECRETARIO:

Dr. Pablo Gustavo Damián Matzumura

Departamento de Biología de la Reproducción, División de Ciencias Biológicas y de la Salud.

Universidad Autónoma Metropolitana Iztapalapa (UAM-I). Ciudad de México, México. Email:

pdamian@izt.uam.mx Work Phone: +52 (55) 6501 9230.

VOCAL / VOCAL

Dr. Javier Esteban Jiménez Salazar

Departamento de Biología de la Reproducción, División de Ciencias Biológicas y de la Salud.

Universidad Autónoma Metropolitana Iztapalapa (UAM-I). Ciudad de México, México. Email:

jejs@xanum.uam.mx Work Phone: +52 (55) 1398 4917.

VOCAL / VOCAL

Dr. Benjamín Pérez Aguilar

Departamento de Ciencias Biológicas y de la Salud. Universidad Autónoma Metropolitana
Iztapalapa (UAM-I). Ciudad de México, México. Email: benjamin.peag@gmail.com Work

Phone: +52 (55) 2311 4108.

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EXTENDED SUMMARY SPANISH / RESUMEN EXTENSO ESPAÑOL

“El GDF11 mitiga la respuesta pro-tumoral en macrófagos M2-like sobre las células derivadas de HCC”. Introducción/antecedentes: El carcinoma hepatocelular (HCC) es un problema de salud mundial. Ocupa el sexto y tercer lugar en incidencia y mortalidad, respectivamente (Abe y Kamimura, 2023; Amin et al., 2025; Chan et al., 2025; Siegel et al., 2024). El HCC puede producirse por infección con virus hepatotrópicos, consumo de alcohol, toxicidad por xenobióticos (Kale et al., 2024; Zheng et al., 2025) e ingesta de dietas enriquecidas con lípidos o fructosa (Chávez-Rodríguez et al., 2025; Simoni-Nieves et al., 2021). La inflamación crónica, como proceso, exacerba la agresividad del tumor (Yu et al., 2018). Los tumores más agresivos poseen alta vascularización e infiltración leucocitaria, incluyendo macrófagos asociados a tumores (TAMs), demostrados en animales con dietas enriquecidas con colesterol (Enríquez-Cortina et al., 2017; Simoni-Nieves et al., 2021). Los TAMs juegan un papel importante en la evolución del HCC y se encuentran en niveles elevados en el microambiente tumoral (TME), formando parte del microambiente inmunitario tumoral (TIME) (Keren et al., 2018; Li et al., 2024). Los macrófagos “M1-like” eliminan patógenos y células transformadas mediante fagocitosis y las especies reactivas de oxígeno (ROS) (Tian et al., 2019; Zheng et al., 2023). Los TAMs o macrófagos “M2-like” (CD206⁺), desencadenan respuestas protumorales e inmunosupresoras (Li et al., 2024; Luo et al., 2024; Yuan et al., 2025). Las interleucinas (IL) como IL-4/13 (Ji et al., 2017; Little et al., 2019) o el factor de crecimiento transformante (TGF)- β provocan una polarización “M2-like” y la promoción tumoral (Zhang et al., 2016). Además, el TGF- β posee efectos pleiotrópicos dentro del TIME (Jin et al., 2022; Ning et al., 2021).

El factor de diferenciación de crecimiento (GDF)-11, miembro de la superfamilia del TGF- β , presenta propiedades antitumorales, a diferencia de TGF- β , particularmente en HCC. El GDF11 usa el mecanismo mediado por las proteínas Smad 2/3 (Gerardo-Ramírez et al., 2023). Además, GDF11 reduce la proliferación, invasión, migración, metabolismo lipídico y bioenergético, promoviendo la transición mesenquimal-epitelial (MET) (Gerardo-Ramírez et al., 2019; Hernández et al., 2021). Ya hemos descrito los efectos en leucemias (Sánchez-Rodríguez M et al., 2025), lo que nos dirige a postular al GDF11 con un enfoque terapéutico contra el núcleo tumoral y sus componentes del TIME, como los TAMs. La propuesta de dos ejes podría representar una innovación para combatir al HCC, en combinación con la inmunoterapia actual u otras terapias dirigidas. En general, esta investigación describe la mitigación de la respuesta protumoral mediada por el GDF11 contra macrófagos “M2-like” y su relación con las células derivadas del HCC.

Justificación: El cáncer es una enfermedad mortal según la OMS (<https://www.who.int/news-room/fact-sheets/detail/cancer>). Los tumores malignos, como el cáncer de hígado, ocupan por su incidencia lugares preocupantes en el mundo actual y se espera un incremento de 1,52 millones de casos nuevos para el año 2050 (Chan et al., 2025). En México ocupan el tercer lugar en las muertes relacionadas con el cáncer. El comunicado de prensa mexicano más reciente reportó que en 2023 se produjeron 799,869 nuevas muertes, de las cuales 11.4 % fueron relacionadas con cáncer; dentro de este, el 52.4 % en mujeres y el 47.6 % en hombres. Cabe destacar que el cáncer de hígado y de las vías biliares intrahepáticas se encuentra entre las cuatro causas de muerte más frecuentes en el país (INEGI, 2025). Además, en México es preocupante la alta ingesta de dietas ricas en lípidos, factor clave para el desarrollo de enfermedad metabólica esteatósica asociada a disfunción metabólica MASLD y esteatohepatitis

asociada a enfermedad metabólica (MASH), los cuales desarrollan subtipos de HCC muy agresivos (Chávez-Rodríguez et al., 2025; Enríquez-Cortina et al., 2017; Simoni-Nieves et al., 2021) y resistentes a la inmunoterapia actual (Pfister et al., 2021). Asimismo, el reclutamiento de TAM dificulta su tratamiento debido a su relación favorable con las células transformadas (Escobedo-Calvario et al., 2022). Un tratamiento más específico con dos ejes, es decir, que destruya las células cancerosas de HCC y revierta la polaridad de los TAMs, sería la terapia ideal para esta enfermedad. Por lo tanto, el abordaje de esta estrategia en modelos experimentales *in vitro* es el primer paso para comprobar su efectividad y posteriormente trabajar con modelos preclínicos.

Hipótesis: El GDF11 también puede reprogramar el TIME, dirigiéndose específicamente a los TAMs con polaridad “M2-like”, reduciendo así su cooperación maligna con las células transformadas de HCC.

Objetivo: Evaluar la respuesta antitumoral del GDF11 en macrófagos “M2-like” en interacción con líneas celulares de HCC.

Objetivos específicos: 1) Evaluar los efectos antitumorales del GDF11 en macrófagos. 2) Evaluar la polaridad de los macrófagos “M2-like” en respuesta a GDF11. 3) Caracterizar el estado metabólico y redox inducido por GDF11 en macrófagos polarizados. 4) Evaluar la respuesta de los macrófagos “M2-like” tratados con GDF11 en la línea celular Huh7. 5) Determinar la secreción de citocinas mediada en macrófagos tratados con GDF11.

Material y métodos: Macrófagos diferenciados con forbol-12-miristato-13-acetato (PMA) fueron tratados con GDF11 recombinante humano (50 ng/ml) cada 24 h. Para la activación de

macrófagos se aplicaron lipopolisacárido (LPS), interferón gamma (IFN- γ), IL-4, IL-13 y TGF- β según la polaridad requerida. Las técnicas o ensayos utilizados en el estudio fueron los siguientes. 1) Manejo de los cultivos celulares de la línea THP-1, derivada de monocitos con capacidad de diferenciarse en células con características de macrófagos, y las células Huh7 derivadas de un hepatoblastoma, tumor hepático. 2) Ensayos de viabilidad y proliferación celular con cristal violeta y CCK-8. 3) Polarización de macrófagos utilizando los estímulos correspondientes de cada polaridad. 4) Tratamientos con GDF11 posteriores a la polaridad específica adquirida en macrófagos. 5) Obtención de macrófagos por métodos no enzimáticos. 6) Citometría de flujo para evaluar la expresión del marcador CD206 en macrófagos. 7) Aislamiento y cuantificación de proteínas. 8) Detección de proteínas específicas por inmunoblot. 9) Determinación metabólica en tiempo real utilizando inhibidores específicos para determinar la tasa de acidificación extracelular (ECAR) y la tasa de consumo de oxígeno (OCR). 10) Detección de ROS y fagocitosis por métodos semicuantitativos usando DHE. 11) Cuantificación de colesterol total por OPA. 12) Evaluación de la migración celular por ensayos de herida-cicatriz. 13) Detección de citocinas en medios condicionados (CM) de macrófagos. 14) Análisis estadístico usando el software GraphPad Prism 8. Todos los datos son presentados como promedio $\pm$ error estándar de la media ($\pm$ SEM) con prueba de análisis de varianza (ANOVA). El valor de $p < 0.05$ fue considerado como significativo.

Resultados y discusión: Nuestro estudio demostró que GDF11 activa la vía canónica de las proteínas Smad 2/3 mediante fosforilación (**Figura 6**), sin efectos citotóxicos ni proliferativos en la línea THP-1 diferenciada a macrófagos (**Figura 6**), tal y como se ha reportado en otros modelos celulares. Además, se logró replicar y estandarizar la polaridad de macrófagos “M2-like” (CD206⁺) mediante el uso de IL-4, IL-13 (**Figura 7**) o medios condicionados (CM) derivados

de células Huh7 (**Figura 8**); sin embargo, se continuó el protocolo con tratamiento con ILs y así tener un modelo más controlado. Más adelante, proporcionamos evidencia de que el GDF11 disminuye la polaridad de los macrófagos “M2-like” en tiempos de 72 h y 144 h, ya que promovió la reducción del marcador CD206 en las poblaciones (**Figura 9**), hecho que sugiere una pérdida de propiedades protumorales en estos leucocitos. Por otro lado, el GDF11 también afectó la bioenergética mitocondrial (**Figura 11**) y el metabolismo de colesterol (**Figura 12**) en los macrófagos, lo que podría afectar respuestas específicas como ocurre con el inmunometabolismo de muchas células blancas. Más adelante, la aplicación de GDF11 indujo cambios en el estado redox y aumento en las especies reactivas de oxígeno (ROS), independientemente de la polaridad, lo que sugiere un fenotipo de macrófagos “M1-like” (**Figura 13**). El mismo incremento se comparó con macrófagos tratados con perlas de látex para simular un proceso de fagocitosis y detección indirecta por ROS (**Figura 13**).

En todos los experimentos se recuperó el CM de macrófagos tratados o no con GDF11, el cual fue aplicado en diferentes diluciones sobre células Huh7, estandarizando la dilución 1:10 de macrófagos “M2-like” como idónea, ya que incrementa la migración de estas células sin afectar la viabilidad (**Figura 14**). Acorde a lo esperado, todos los CM derivados de macrófagos tratados con GDF11 redujeron los parámetros de agresividad tales como proliferación y migración comparados con el control no tratado (NT) (**Figuras 15 y 16**). Los datos muestran, más que disminuir, un proceso de neutralización de los parámetros mencionados, es decir, retrasa el incremento del número de células y la cicatrización de la herida. Subsecuentemente, para demostrar que este efecto fue debido a la secreción del macrófago y no a posibles restos de GDF11, utilizamos un anti-GDF11 como control. Los resultados mostraron que los parámetros de agresividad de igual manera fueron retardados sobre las células Huh7 (**Figura 17**).

Finalmente, nosotros evaluamos el contenido de los CM usando un kit de detección específica de 42 citocinas, las cuales se encontraron alteradas por el tratamiento con GDF11 (**Figura 20**). Esto demuestra que esta molécula modifica los efectos protumorales de los TAMs o macrófagos “M2-like”, y que a su vez compromete el comportamiento de las células derivadas de HCC.

Conclusión: Nuestro estudio *in vitro* demostró que los macrófagos “M2-like” mediante la aplicación de rGDF11 reducen y alteran sus efectos protumorales, lo que podría convertirse en una estrategia eficaz para controlar o mitigar la agresividad de los tumores sólidos, incluido el HCC.

ABSTRACT / RESUMEN

Introduction. Hepatocellular carcinoma (HCC) represents one of the most aggressive cancers worldwide. Chronic inflammation supports tumor aggressivity and evolution, and the infiltration of specific leukocytes, such as tumor-associated macrophages (TAMs), also known as M2-like macrophages, which they associated with the most aggressive behavior. M2-like macrophages execute the growth and migratory capacity of cancer cells, including those of HCC. Contemporary therapies are not well optimized for abolishing transformed cells or counteracting the tumor immune microenvironment (TIME) leukocytes, especially TAMs. Growth differentiation factor 11 (GDF11) could represent an optimistic dual therapeutic target owing to its reported anti-tumorigenic and immunomodulatory properties. **Aim.** To characterize the effects of GDF11 in M2-like macrophages and the HCC cell interaction using a functional *in vitro* model. **Methodology.** The research used THP-1 and Huh7 cell lines. Recombinant GDF11 (50 ng/mL) was applied every 24 h on THP-1 differentiated macrophages with M2-like polarization employing IL-4 and IL-13. Firstly, the effects of GDF11 on signaling, viability, proliferation, phenotype, morphology, metabolism, and redox state in macrophages were characterized. Thereafter, conditioned media (CM) were extracted from macrophages, and indirect co-cultures were performed using Huh7 cells. The key functional parameters were assessed through proliferation and migration assays. Finally, we characterized the content of the CM using the cytokine array membrane assay. **Results.** Our study demonstrated that GDF11 activates the canonical pathway Smad 2/3 without cytotoxic or proliferative effects. Moreover, we provide evidence that GDF11 diminishes the pro-tumoral features of M2-like macrophages. GDF11 promotes the reduction of the M2 macrophage marker, cluster of differentiation 206 (CD206), suggesting a loss of pro-tumoral properties in these leukocytes. Furthermore, GDF11 induced

changes in metabolism and a climb in reactive oxygen species (ROS). Extraction of CM derived from GDF11-treated M2-like macrophages revealed a reduction in the proliferation and migratory capacity of liver cancer cells. Furthermore, the cytokine profile was altered by GDF11 application, exhibiting that this molecule modifies the pro-tumoral effects of TAMs, which in turn impact the behavior of HCC-derived cells. **Conclusions.** This *in vitro* study suggests that mitigating tumor-promoting factors, such as M2-like macrophages, by applying GDF11 may become an effective strategy for controlling or mitigating the aggressiveness of solid tumors, including HCC.

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1. INTRODUCTION

Hepatocellular carcinoma (HCC) constitutes a serious health issue worldwide, considered one of the most aggressive among liver cancer subtypes. HCC is classified as the sixth and third leading cause of incidence and mortality, respectively (Abe & Kamimura, 2023; Amin et al., 2025; Chan et al., 2025; Siegel et al., 2024). HCC emerges from hepatotropic virus infection, alcohol consumption, xenobiotic toxicity (Kale et al., 2024; Zheng et al., 2025), or by the uptake of enriched-lipid or fructose diets (Chávez-Rodríguez et al., 2025; Simoni-Nieves et al., 2021). Independent of tumor etiology, this leads to chronic inflammation, which exacerbates tumor development and aggressiveness (Yu et al., 2018).

It has been broadly reported that the most aggressive liver tumors exhibit increased vascularization and elevated leukocyte infiltration, including tumor-associated macrophages (TAMs), which is associated with the worst prognosis, as observed in cholesterol-enriched diet models (Enríquez-Cortina et al., 2017; Simoni-Nieves et al., 2021). This indicates that TAMs are a meaningful component of the tumor microenvironment (TME), particularly in the tumor immune microenvironment (TIME), which contributes to the aggressiveness of many tumors, including HCC (Keren et al., 2018; Li et al., 2024). Macrophages belong to the innate immunity, executing specific responses such as eliminating pathogens and cancerous cells through phagocytosis, a mechanism that depends on the production of reactive oxygen species (ROS) and additional processes (Tian et al., 2019; Zheng et al., 2023). In the context of cancer, TAMs, also known as M2-like macrophages (CD206⁺), are inclined to be activated in this alternative way, triggering pro-tumor and immunosuppressive responses, in contrast to M1-like or classically activated macrophages, which induce anti-tumor effects (Li et al., 2024; Luo et al., 2024; Yuan et al.,

2025). High interleukin (IL) levels in the tumor, such as IL-4/13 (Ji et al., 2017; Little et al., 2019) or transforming growth factor β (TGF- β), lead to M2-like polarization and tumor promotion (Zhang et al., 2016). Besides, TGF- β confers aggressive characteristics to the tumor and its microenvironmental components of TIME (Jin et al., 2022; Ning et al., 2021).

We formerly reported that growth differentiation factor 11 (GDF11), a member of the TGF- β superfamily, displays tumor suppressor properties, opposed to TGF- β , particularly in HCC-derived cells by diminishing aggressiveness through a mechanism of canonical signaling mediated by Smad 2/3 proteins (Gerardo-Ramirez et al., 2023). Moreover, GDF11 reduces proliferation, invasion, and migration, fostering a mesenchymal-to-epithelial transition (MET) (Gerardo-Ramírez et al., 2019) and lessens lipid and bioenergetic metabolism (Hernandez et al., 2021). The therapeutic approaches for HCC could target cancer cells and the TIME components. Our hypothesis is that there is a narrow relationship between GDF11 and the mitigation or neutralization of pro-tumoral effects in leukocytes from TIME, such as TAMs, as we published in cancer cells. The two-axis proposal could represent an innovative strategy to attack HCC aggressiveness in conjunction with current immunotherapy and other targeted therapies. In general, this research described how GDF11 mitigates the pro-tumoral response *in vitro* against M2-like differentiated macrophages and their relationship with HCC-derived cells in the context of aggressive behavior.

2. BACKGROUND IN THE RESEARCH FIELD.

2. 1. The liver is a multifunctional organ.

The liver is a complex organ that fulfills a variety of roles where hepatocytes promote albumin production, glycogen storage, lipid and cholesterol metabolism, xenobiotic detoxification,

endotoxin removal, anti-microbial protein production, acute phase response, clotting factor production, complement protein production, and antigen presentation, demonstrating non only metabolic roles but also immunological processes making it a multifunctional organ (Robinson et al., 2016). The liver is composed of hepatocytes (60-80%), which represent the parenchymal cells, and non-parenchymal cells (20-40%). The non-parenchymal cells are formed by endothelial cells (EC), also called liver sinusoidal endothelial cells (~50%), Kupffer cells (KC) or liver macrophages (~20%), many subclasses of lymphocytes (~25%), and hepatic stellate cells (~8%) forming and specifically distributed in the liver sinusoids of hepatic lobule (Freitas-Lopes et al., 2017; Terai et al., 2017). The liver is always exposed to damage by different hazard insults, in a constant homeostasis, from injury to resolution, but if it is not controlled, it can be triggered to chronic inflammation with minimal resolution, spanning the entire spectrum from a healthy liver to fatty liver, fibrosis, cirrhosis, and liver cancer as the last result (Matchett et al., 2024; Robinson et al., 2016; Yu et al., 2018).

2. 2. Cancer definitions.

Cancer is a lethal disease worldwide, according to the World Health Organization (WHO). There are numerous definitions of cancer in the literature, and their meanings have evolved over time. The old definitions going from *“A tumor is an autonomous new growth of tissue”* (Neoplastic Disease – a treatise on tumors, Erwing, 1919) to a new definition as *“Cancer isn’t a single disease. Cancer is a group of diseases characterized by the uncontrolled proliferation of cells. Ignoring the body’s signal to stop, malignant cells multiply to form tumors in organs and tissues or, in the case of blood cancers, crowd out normal cells in the bloodstream and bone marrow”* (American Association for Cancer Research, 2023). Some authors described in a modern

definition as “*Cancer is a disease of uncontrolled proliferation by transformed cells subject to evolution by natural selection.*” The term “transformed” encompasses the Hallmarks of Cancer, including the ability to invade and metastasize to many sites in the body; and “evolution by natural selection” modernizes the definition to include the genetic, epigenetic changes and tumor microenvironment (TME) adaptation that lead to the lethal phenotype (Brown et al., 2023).

2. 3. Epidemiology of liver cancer and classification.

Liver cancer represents a severe health problem worldwide and is defined as a highly heterogeneous malignant disease due to its morphological and molecular diversity (Li & Wang, 2016; Siegel et al., 2024). Liver tumors include benign (non-cancerous) and malignant (cancerous) versions; the latter are classified as primary liver tumors (PLC) with liver origin and metastatic liver tumors or extrahepatic cancers (Abe & Kamimura, 2023). Liver malignant tumors are ranked as the sixth most common cancer and the third cause of cancer-related mortality worldwide. In 2022, liver cancer accounted for 0.87 million new cases and 0.76 million deaths. It is expected that the incidence could increase to 1.52 million new cases in 2050 (Chan et al., 2025). In Figure 1, illustrations of incidence and mortality worldwide are presented from the Global Cancer Observatory website. In Mexico, malignant tumors are in third place as the main cause of death behind heart problems and diabetes mellitus, in first and second place, respectively. According to a Mexican press release, in 2023, 799,869 new deaths occurred, of which 91,562 (11.4 %) were new cancer-related deaths, 52.4% in women and 47.6% in men. Liver and intrahepatic bile duct cancers were among the four most frequent cancer-related deaths in this country (INEGI, 2025).

Liver tumors encompass a wide variety of subtypes, with hepatocellular carcinoma (HCC) being the most common case (75-80%), followed by intrahepatic cholangiocarcinoma (10-15%) as bile duct cancer (Amin et al., 2025). The other uncommon hepatic tumors with minor cases include lymphomas, combined hepatocellular and cholangiocarcinoma, angiosarcoma, inflammatory pseudotumor, hepatoblastoma, angiomyolipoma, intraductal papillary neoplasm of the bile duct, mucinous cystic neoplasm, hepatic epithelioid hemangioendothelioma, mesenchymal hamartoma, IPT-like FDC sarcoma, and Hydatid cyst (Wu et al., 2016).

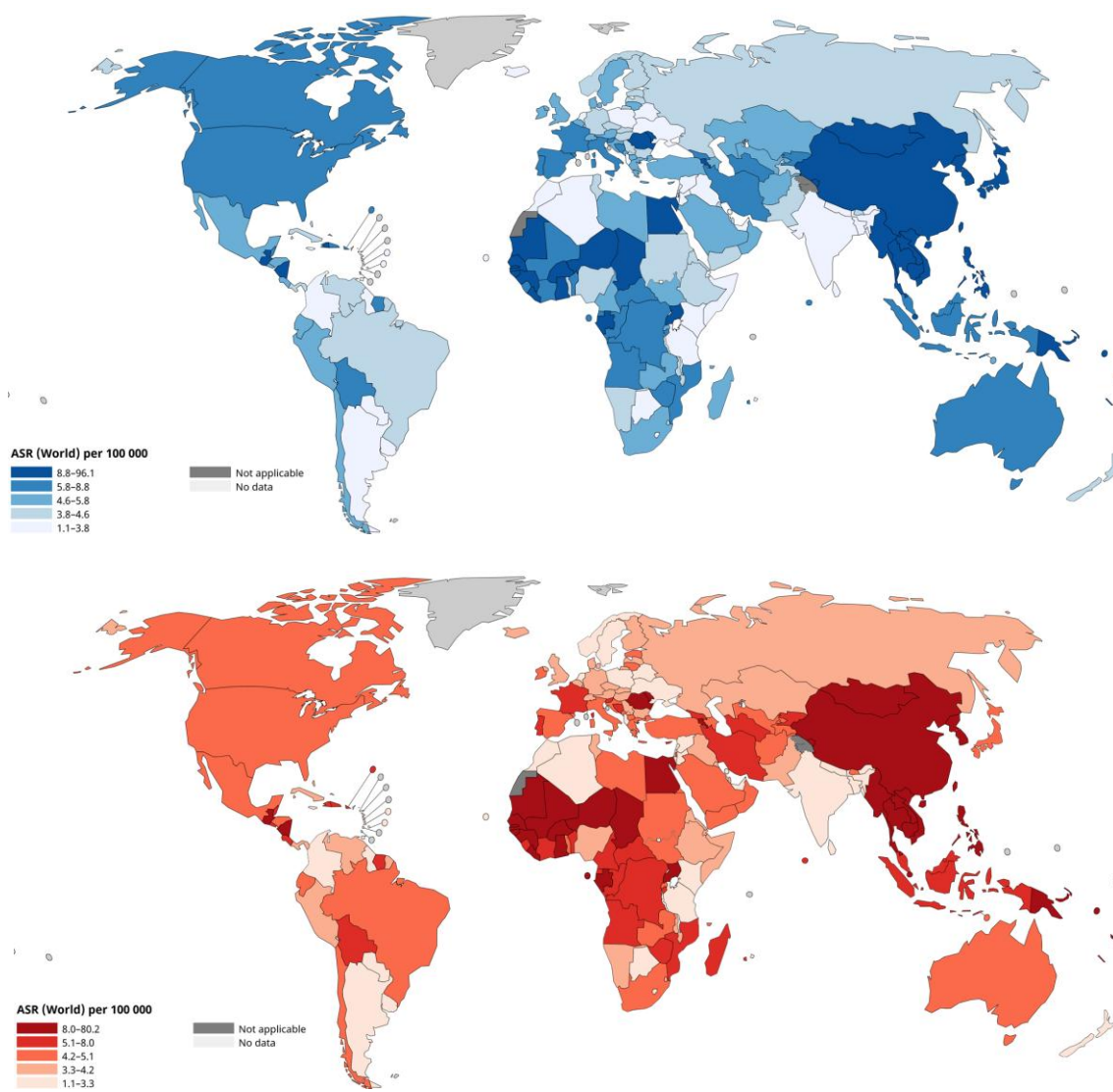


Figure 1. Liver cancer distribution worldwide. Heatmap of global incidence (up, blue scale) and mortality (down, red scale) of both sexes of the liver and intrahepatic bile duct cancer in 2022 (last updated). The numbers are per 100,000 people a year. Information and illustrations were taken from the GLOBOCAN website (<https://gco.iarc.fr/en>).

2. 4. HCC etiologies and risk factors.

HCC is considered one of the most aggressive liver cancer subtypes in the world (Zheng et al., 2025). The etiology or risk factors of HCC cancer are due to the hepatotropic viral infections (hepatitis B and C viruses), alcohol, xenobiotics, smoking, fatty liver, genetic mutations, aflatoxin, obesity, and metabolic factors (Kale et al., 2024). Currently, it is well established that diets enriched in lipids or fructose are promoters of the most aggressive HCC tumor subtypes (Chávez-Rodríguez et al., 2025; Chávez-Rodríguez et al., 2021; Simoni-Nieves et al., 2021). After liver injury, fibrosis and cirrhosis could lead to HCC or could lead directly from metabolic dysfunction-associated steatohepatitis (MASH) that is developed from Metabolic dysfunction-associated steatotic liver disease (MASLD), formerly known as non-alcoholic fatty liver disease (Matchett et al., 2024; Phoolchand & Khakoo, 2024). MASLD is the leading chronic liver disease, affecting 30% of adults, linked to metabolic syndrome, obesity, type 2 diabetes, and cardiometabolic issues (Armandi et al., 2025). Strikingly, MASH-HCC tumors are considered extremely aggressive due to paradoxical response using monoclonal antibodies as immunotherapy; the inflammation, fibrosis, and tumorigenesis are exacerbated (Peng et al., 2021; Pfister et al., 2021).

2. 5. Chronic inflammation and hepatocarcinogenesis.

Inflammation is a pivotal physiological process in the organism that works as the first line of defense against harmful signals, damaged cells, and foreign substances, including pathogens (Chavda et al., 2024; Maddipati, 2024). Moreover, inflammation involves the recruitment and modeling of immune cells or leukocytes, blood vessels, and the release of pro-inflammatory secretory molecules. The normal process, known as acute inflammation, is a natural mechanism for healing and recovery. Meanwhile, chronic inflammation, also known as non-resolving inflammation, represents the incomplete resolution of acute inflammation and is a cause that alters homeostasis through pathology, leading to carcinogenesis (Chavda et al., 2024; Maddipati, 2024; Yu et al., 2018). Chronic inflammation is responsible for inducing colorectal cancer from chronic inflammatory disease, or liver cancer from chronic hepatitis and MASLD, as general examples (De Visser & Joyce, 2023).

In the case of the liver, it also transitions from inflammation to resolution in response to acute hazard insults, such as hypoxia, ER stress, lipid accumulation, endotoxins, reactive oxygen species (ROS), etc. are also responsible for inducing HCC; however, uncontrolled stimuli or constant damage can trigger these chronic effects. For example, in MASLD progression to MASH, there are 3 steps: 1) Hepatic stress sensing; 2) Inflammation, accompanied by steatosis and dead cells; and 3) Escalation in magnitude, followed by fibrosis and cirrhosis (Mladenović et al., 2024). The escalation could lead to altering the resolution phase (Barnig et al., 2019; Mohammad-Rafiei et al., 2024). Non-resolving inflammation is driven by canonical signaling pathways and well-identified transcription factors, such as NF- κ B and STAT3, in many tissues, including the liver. Non-resolving inflammation has been included as a relevant hallmark of

cancer that substantially contributes to the development and progression of cancer, such as HCC (Yu et al., 2018).

Tumor-supportive inflammatory media may cause tissue injury, mutagenesis, endothelial dysfunction, angiogenesis, immunosuppression, and matrix remodeling, favoring the tumor onset (De Visser & Joyce, 2023). The most aggressive tumors, such as HCC, exhibit an increase in vascularization and high leukocyte infiltration, such as tumor-associated macrophages (TAMs), which are correlated with the worst prognosis, as has been observed in cholesterol/lipid-enriched diet models as evidence of TME support (Enríquez-Cortina et al., 2017; Simoni-Nieves et al., 2021).

Finally, the inflammation process is accompanied by small ($\approx 6\text{--}70$ kDa) and soluble proteins, called cytokines, derived from both immune and non-immune cells in an autocrine, paracrine, and systemic manner. These cytokines serve as critical mediators that oversee and regulate immune and inflammatory responses. They exhibit pleiotropic effects and are classified based on their type (pro- or anti-inflammatory), cell sources, and biological functions (Liu et al., 2021). The most common and basic classification of cytokines is represented by 1) interleukins (IL); 2) chemokines. 3) interferons (IFN); 4) tumor necrosis factor (TNF); colony-stimulating factors (CSF); and transforming growth factor (TGF)- β (Kupsa et al., 2012). The cytokines in cancer biology vary across tumor types and stages, serving as indicators of aggressivity and acting as either pro- or anti-tumoral factors (Berraondo et al., 2019; Kureshi & Dougan, 2025; Liudahl & Coussens, 2018).

2. 6. Tumor microenvironment (TME) components.

Cancer is a progressive disease that represents a complex micro-ecosystem comprising diverse clones of transformed cancerous cells (Kloosterman & Akkari, 2023; Laplane, 2024) and a heterogeneous group of non-cancerous cells embedded in an altered extracellular matrix and a high variety of molecules, which form the tumor microenvironment (TME) (De Visser & Joyce, 2023). TME formation is cancer-type dependent and plays a relevant role in tumor aggressivity and progression. TME includes diverse immune cell types, cancer-associated fibroblasts (CAFs), blood vascular endothelial cells (EC), lymphatic EC, adipocytes, pericytes, and various additional tissue-resident cells (De Visser & Joyce, 2023). Recent evidence has added that neurons and nerves are also integral to TME, and some of the responsible parties for inducing metastasis through various mechanisms (De Visser & Joyce, 2023; Hoover et al., 2025; Padmanaban et al., 2024). On the other hand, the microbiome plays a significant role as an integral part of the TME (Ciernikova et al., 2022). This has been observed in the tumor-resident intracellular microbiome, which promotes aggressivity through metastatic colonization (Fu et al., 2022). The cellular and molecular composition of the TME depends on the organ in which the tumor arises, patient characteristics, and the tumor stage, making it a space-time disease rather than a purely genetic one (De Visser & Joyce, 2023).

The function of immune system cells is high, and they can eliminate transformed cells; however, tumors gradually shape or immuno-evade these leukocytes into an immunosuppressive phenotype (Lv et al., 2022). Regarding immune cells called tumor-infiltrated leukocytes (TILs) in the TME that takes part of chronic inflammation (Chen et al., 2018), there are three types: adaptive immune cells: CD8⁺ T (cytotoxic lymphocytes, CTLs) cells, CD4⁺ T (effector) cells, T regulatory (Treg) cells, and B cells; myeloid immune cells: monocytes, macrophages,

neutrophils, dendritic cells (DCs), mast cells, eosinophils, myeloid-derived suppressors cells (MDSC), and platelets; immune cells at the interface of adaptive and innate immunity: natural killer (NK) cells, invariant NK T (iNKT) cells, gamma delta T ($T\gamma\delta$) cells, and innate-like lymphocytes (ILC) (De Visser & Joyce, 2023). This TILs' diversity is an integral component of the TME and has been found to correlate with prognosis and response to different therapies (Chen et al., 2018). Currently, this type of TME is referred to as the tumor immune microenvironment (TIME), characterized by high diversity and classifications that depend on current immunotherapy (Binnewies et al., 2018; Lv et al., 2022).

There are three classes of TIMEs according to leukocyte distributions: 1) infiltrated-excluded TIMEs or cold tumors, characterized by the exclusion of CTLs and tumor-associated macrophages (TAMs) from the tumor core, with low immunogenicity; 2) infiltrated-inflamed or hot tumors, which are defined by an abundance of programmed cell death ligand 1 (PD-L1) expression on cancer and myeloid cells and high CTLs blended in the core; and 3) infiltrated-tertiary lymphoid structures (TLSs) TIMEs, containing TLSs, with a composition similar to lymph nodes, including B cells, DC, and Treg cells (Binnewies et al., 2018). Finally, the content and distribution of not only vary between tumor types, but also between patients (Keren et al., 2018).

2. 7. TIME and immune responses in the liver.

Despite having enough evidence, the HCC TIME is poorly characterized. Research focusing on RNA sequencing (RNA-seq) has revealed significant differences in the transcriptome of HCC TIME compared to normal or healthy liver tissue. The evidence indicates that HCC etiology and tumor stage are not linked to major changes in the immune contexture; however, the analysis has revealed a reduction in genes involved in B cells, T cells, CD8+ cells, CTLs, T helper (Th) 1

responses, $\gamma\delta$ T cells, NK cells, DCs, and type II interferons. On the other hand, the increment was in genes involved in Th2 responses, Treg cells, plasmacytoid dendritic cells, and macrophages (Foerster et al., 2018). Additional studies using integrated single-cell RNA-seq with a magnifying glass, revealing a high diversity of immune cell subsets at five immune sites: lymph nodes, blood, ascites, adjacent liver, and tumor, in the context of HCC. Notably, myeloid cells, such as LAMP3⁺ DCs, possess the potential to migrate to the tumor, and additionally, TAMs exhibit distinct transcriptional states and are associated with a poor prognosis (Zhang et al., 2019). Moreover, similar techniques, demonstrated TAMs are largely responsible for inducing an immunosuppressive and exhausted status of T cells (Ho et al., 2021)

The specific immune responses that modulate the mitigation or exacerbation of tumor aggressiveness in the liver and other organs are also mediated by effector CD4⁺ T helper (Th) subsets, such as Th1, Th2, Th9, Th17, Th22, T follicular helper, and Treg (Liudahl & Coussens, 2018). There is an important balance between Th1/Th2 in the context of tumor progression. Th1 provides immunostimulation and tumor regression through the secretion of IL-2, IFN- γ , TNF- α , and TNF- β , whereas Th2 induces downregulation of immunity and tumor promotion via the secretion of IL-4, IL-5, IL-6, IL-10, and IL-13 (Ji et al., 2017; Lin et al., 2017). In HCC, Th2 responses predominate over Th1 responses, as observed in patients (Ji et al., 2017). Furthermore, more evidence indicates that Th17, Th22, and Treg also predominate as protumoral responses, contrary to Th1, which is typically considered antitumoral (Liudahl & Coussens, 2018).

2. 8. Tumor-associated macrophages (TAMs) relevance.

2. 8. 1. The macrophage and its physiological roles.

Macrophages belong to the innate immune system and were defined by Ilya Metchnikov as phagocytic cells. They exhibit high plasticity, developing pleiotropic functions in a spatiotemporal manner and in response to the needs of the microenvironment (Luo et al., 2024). Macrophages distributed in the body tissues have three sources: 1) yolk sacs and fetal liver as prenatal embryonic precursors, the origin of resident macrophages; 2) macrophage/dendritic cell progenitors; and 3) differentiated from monocytes produced in bone marrow (Yuan et al., 2025). Moreover, they promote self-renewal to support the tissue, as occurs in the liver (Krenkel & Tacke, 2017).

2. 8. 2. The macrophage polarization.

Resting macrophages (M0) exhibit activation, leading to classical (M1) or M1-like macrophage polarization in response to IFN- γ , LPS, TNF- α , IL-12, IL-23, and GM-CSF stimuli. This polarization is responsible for pro-inflammatory responses, microbicidal activity, ROS production, and undesirable tissue damage during inflammation. In contrast, alternative activation (M2) leads to M2-like macrophage polarization induced mainly by IL-4, IL-13, or IL-10, characterized in general by anti-inflammatory activity, immunosuppressive response, pro-tumorigenic functions, as well as tissue remodeling and repair through phagocytosis via efferocytosis, and vascularization (Chen et al., 2019; Li et al., 2024; Luo et al., 2024; Yuan et al., 2025). Moreover, M2 activation is classified into four subphenotypes: 1) M2a, induced by IL-4 and IL-13, which promotes wound-healing, fibrosis, and cell growth; 2) M2b also known as TAM, induced by immune complex, TLR, and IL-1 β , which triggers anti-inflammatory and immunosuppressive properties; 3) M2c, induced by glucocorticoids, IL-10, and TGF- β , which

performs phagocytosis, immunosuppression, angiogenesis, and tissue fibrosis development; and 4) M2d, induced by $A_{2A}R$, which promotes angiogenesis and tumor promotion (Luo et al., 2024; Yuan et al., 2025). Figure 2 illustrates the general characteristics of both polarities.

These two phenotype classifications have been established to explain the transcriptional and functional states identified *in vitro*, compared to the heterogeneity observed *in vivo* (Blériot et al., 2024). Both macrophage types coexist in a dynamic balance, under normal and pathological circumstances, to lead the response. Finally, a new nomenclature is based on the shared genes from M1 and M2, as observed in M4 macrophages. (Lampiasi, 2023).

2. 8. 3. The nomenclature dilemma in macrophage polarization.

Intriguingly, M1 and M2 nomenclature had been employed to define the “supposed” two opposite macrophages; however, this oversimplified concept was not fit within the high-spectrum subsets. Moreover, the physiological and pathological conditions of macrophages represent a continuum, exhibiting extreme plasticity and heterogeneity. Therefore, polarized macrophages have recently been described as “M1-like” and “M2-like,” referring to the M1 and M2 phenotypes, respectively, according to the declared clarifications. Consequently, for M1-like acquisition, diverse signals are required, and not limited to LPS or IFN- γ , to induce cytotoxic phagocytes. Accordingly, another blend of myriad factors is required for the M2-like sub-phenotypes, independent of IL-4 or IL-13 (Parisi et al., 2018; Xue et al., 2024).

2. 8. 4. Macrophage markers.

M1-like macrophages express iNOS and membrane markers, including MHC-II, TLR-2, TLR-4, CD80, and CD86. In contrast, M2-like macrophages express CD163, CD206, CD209, FIZZ1,

and Ym1/2 as membrane markers (Li et al., 2024). Additionally, due to the similarity in activities between M2 phenotypes and TAMs, they share the expression of common markers, such as CD163, CD206, and arginase 1 (Arg1), as well as the secretion of proteins like IL-10 and TGF- β (Chen et al., 2019). Figure 2 provides additional information on macrophage markers.

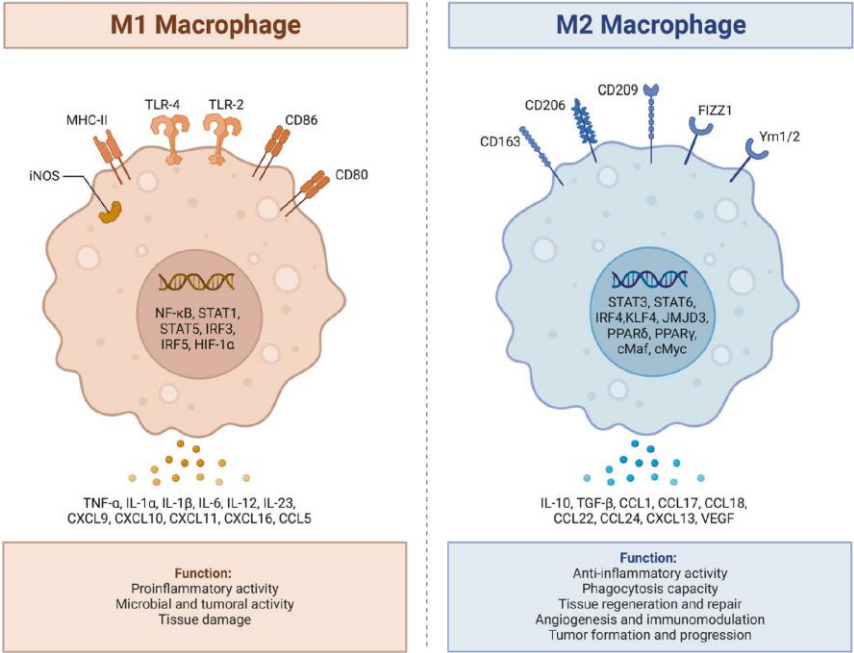


Figure 2. Generalities of macrophage polarization. On the left, M1 macrophage markers, secretion, and functions; on the right, M2 macrophage markers, secretion, and functions. Original figure taken from (Li et al., 2024).

2. 8. 5. Roles of TAMs in cancer aggressivity.

The macrophages coexist with a myriad of cell types in the TME, and are often the most abundant, responsible for tumor development (Blériot et al., 2024; Chen et al., 2019; Keren et al., 2018). TAM functions include tumor growth, tumor migration, tumor invasion, tumor metastasis, tumor angiogenesis, tumor tissue remodeling, maintaining cancer stem cell niche, and immunomodulation to immunosuppression (Chen et al., 2019; Quaranta et al., 2024).

Additional roles describe TAMs as responsible for activating dormancy in disseminated tumor cells, thereby improving the invasion and metastasis of primary tumors (Borriello et al., 2022). In Figure 3, the TAM functions in the TME are illustrated, with additional information highlighted in each process.

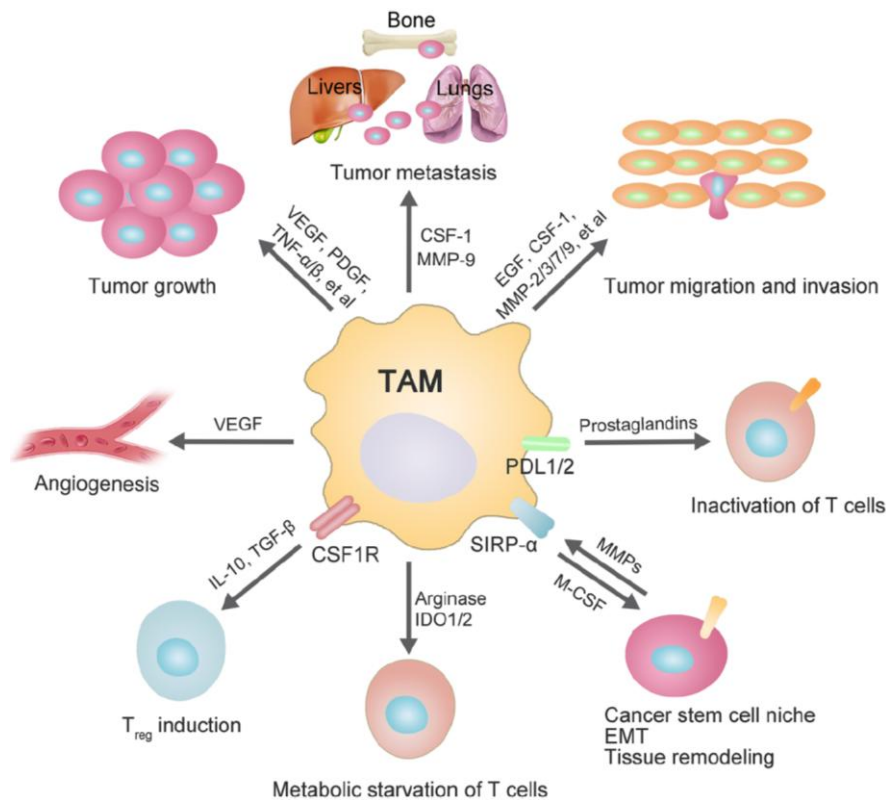


Figure 3. Tumor-associated macrophage (TAMs) roles. TAMs inside TME signals performed a variety of functions to facilitate tumor growth and progression. Original figure taken from (Chen et al., 2019).

2. 8. 6. TAMs in liver cancer.

Hepatic macrophages, called KCs, were discovered by Karl Wilhelm von Kupffer in 1876 (Graham et al., 2025). KCs exhibiting heterogeneous types according to their position within the liver lobule (R. Liu et al., 2023). In addition to resident KCs, there are monocyte-derived KCs, monocyte-derived macrophages (MoMFs), liver capsular macrophages, lipid-associated macrophages, splenic monocyte-macrophages, and peritoneal macrophages, with

spatiotemporal roles in homeostasis and disease, including liver cancer (De Ponti et al., 2025; Krenkel & Tacke, 2017; R. Liu et al., 2023). KCs comprise 80-90% of all macrophages in the body and cover up to 20% of non-parenchymal cells in the liver (Terai et al., 2017). In cancer, TAMs can comprise up to 50% of the tumor mass, depending on the type and stage (Wang et al., 2024).

Report shows the notably low presence of resident KCs at the onset of cancer, and further decreases in intermediate and late stages, whereas monocytes and MoMF increase from early or intermediate stages, and further exacerbate in later stages in DEN- and NASH-induced HCC models, revealing dynamics in the macrophage population (Degroote et al., 2021). This fact represents the evolution from pre-TAM to TAM in the premalignancy/onset and tumor progression, respectively (Blériot et al., 2024). NASH-HCC exhibits higher aggressiveness compared to other HCC types (Pfister et al., 2021), carried out by TAM and the upregulation of anti-inflammatory and/or immunomodulatory genes in these cells (Degroote et al., 2021). In addition to the general processes of TAMs, liver TAMs engage in specific cell interactions to inactivate CTLs and NK cells, induce Treg cells, recruit tumor-associated neutrophils, and modulate LCEC and cancer-associated fibroblasts (Quaranta et al., 2024).

2. 8. 7. The immunometabolism in macrophages

Classical M1 macrophages utilize glycolytic pathways and reduce mitochondrial activity to produce energy. Conversely, alternative M2 macrophages exhibit high mitochondrial oxidative phosphorylation (OXPHOS) and spare respiratory capacity. (Van den Bossche et al., 2015). Additionally, M2-like cells perform fatty acid oxidation (FAO) via β -oxidation in mitochondria, a specific process to promote aggressivity in HCC cell lines (Zhang et al., 2018). However,

specifically TAMs are inside of a modified, low-nutrient, hypoxic, and acidic TME, a fact that forces the induction of changes in metabolism, specifically M2-like polarity (Wang et al., 2020). As an example, the metabolism of lactic acid from TME induces an epigenetic effect in macrophages through H3K27 histone acetylation, which switches off pro-inflammatory responses and leads to an immunosuppressive gene program (Shi et al., 2024). On the other hand, low glucose levels (utilized by cancer cells) induce transcriptional changes in macrophages (Wang et al., 2022). However, the evidence is currently controversial due to the capacity of macrophages to adapt and capture glucose (Shi et al., 2022). Moreover, the low oxygen pressure induces the activation of HIF- α and VEGF, leading to angiogenesis characteristic of M2-like polarization (Zhang et al., 2022). Not only macrophages, but also many leukocytes, are strongly associated with immunometabolism, exhibiting specialized responses in microenvironments (Angajala et al., 2018).

2. 8. 8. Cholesterol metabolism in cancer and TAMs

Lipids are essential in cells, including cholesterol metabolism. Cholesterol is used as a precursor of vitamin D, a plasma lipoprotein constituent, a steroid hormone precursor, for bile acid synthesis, to insulate nerve fibers, for membrane fluidity, for membrane homeostasis, and for lipid raft formation (Mayengbam et al., 2021). Also, cholesterol serves as a signaler and is described as promoting the appearance of highly aggressive liver tumors, which are more prominent and highly vascularized (Enríquez-Cortina et al., 2017; Simoni-Nieves et al., 2021). There are three cholesterol sources that provide cancer cells with the aggressive phenotype: 1) dietary cholesterol intake; 2) de novo biosynthesis through the mevalonate pathway in cancer

cells; and 3) taking it from microenvironment cells such as macrophages (Escobedo-Calvario et al., 2022; Goossens et al., 2019).

2. 9. Current therapies and immunotherapies against HCC

For several years, liver cancer has been treated with multiple drugs, including the immune-based therapies, and we agree that the latter represent great relevance according to the TIME-induced aggressivity. The last, including monoclonal antibodies, vaccines, cytokine-induced natural killer cells, T cells transduced with a chimeric antigen receptor (CAR), and TCR-transduced T cells (Cabibbo et al., 2025; Greten & Sangro, 2018). However, some possess secondary effects and are not expected to be efficient, as has been seen in some liver cancers (Greten & Sangro, 2018; Pfister et al., 2021).

More specific therapies against HCC in the context of TAM have been proposed and could represent a good strategy. Some of which include many points: 1) inhibition of monocyte recruitment, which reduces the entrance of MoMF; 2) eliminating TAMs, including resident and derived from MoMF; 3) re-educating TAMs, from M2-like to M1-like polarization to guarantee the tumor reduction; and 4) neutralizing products derived from TAMs using inhibitors or monoclonal antibodies (Bai et al., 2024; Tian et al., 2019). State-of-the-art therapies are tested in CAR-Macrophages in preclinical models for many tumors, including HCC cells (Guan et al., 2024; Pierini et al., 2025).

2. 10. The growth differentiation factor 11 (GDF11)

2. 10. 1. Generalities of GDF11.

Growth differentiation factor 11 (GDF11), also known as bone morphogenetic protein 11 (BMP11), is a key member of the TGF- β superfamily. GDF11 was discovered by the group of Professor McPherron in 1999. Many functions have been associated with this molecule, but the first attributed to GDF11 was as a determinant factor in patterning the axial skeleton (Gerardo-Ramirez et al., 2023; Simoni-Nieves et al., 2019).

In humans, the location of the GDF11 gene (*Gdf11*, *ENSG00000135414*) is in chromosome 12 (12q13.2) with two mRNA splicing variants, GDF11-201 (ENST00000257868.9), and GDF11-202 (ENST00000546799.1). The main splice transcript produces an immature protein (inactive form), also known as the unprocessed pre-pro complex. Specific proteases belonging to furin-type convertases lead to cleavage, generating two 37 kDa peptides (inactive latent complex). Later, the protein convertase subtilisin/kexin 5 (PCSK5) is the enzyme responsible for the cleavage to produce the 25 kDa dimer (mature and functional form), with biologic and physiological effects in multiple organs and cell types, according to the needs of the tissue (Gerardo-Ramirez et al., 2023; Simoni-Nieves et al., 2019).

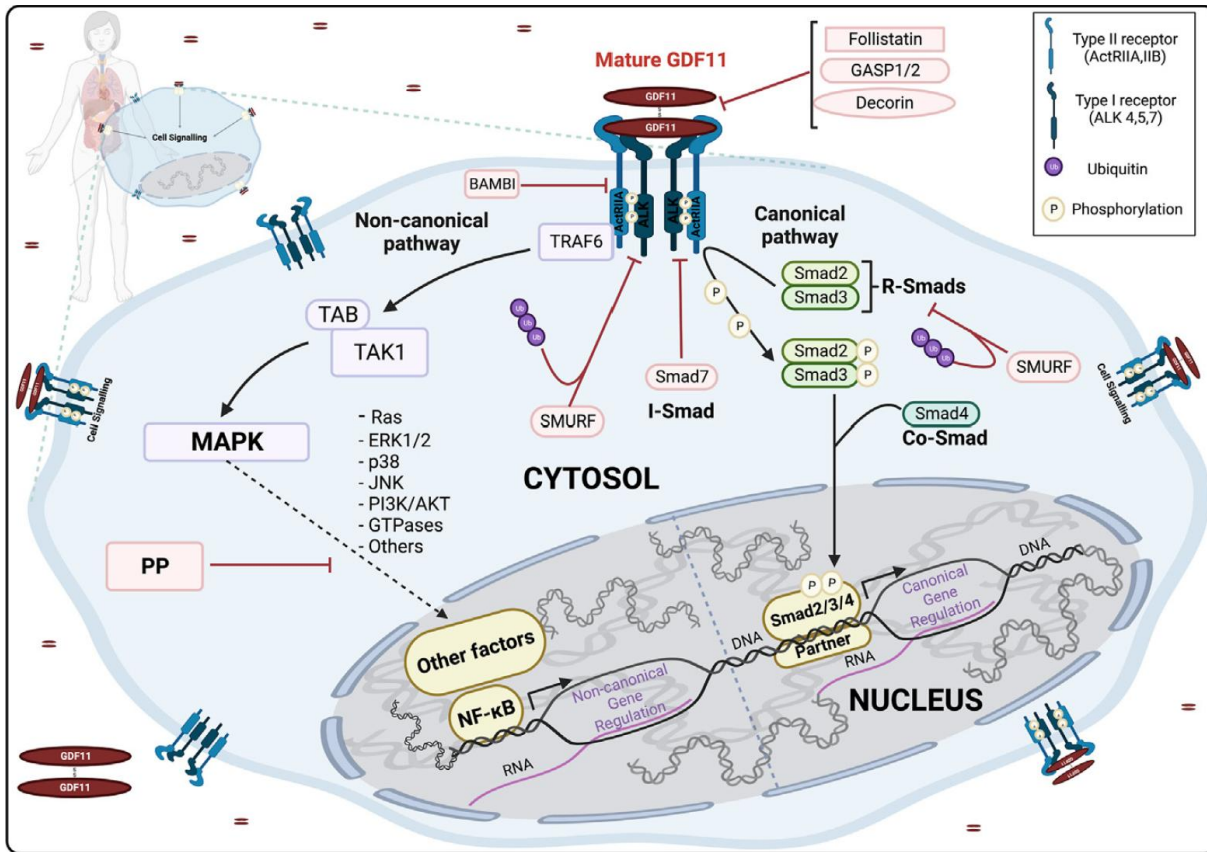


Figure 4. GDF11 signaling pathways. The summary of canonical and non-canonical routes activated by the mature form of GDF11 in the cells. Image was taken from (Gerardo-Ramirez et al., 2023).

GDF11 canonically transduces using Smad proteins. On the membrane, the type II receptor (ActRIIA, IIB) dimerizes, leading to tetramerization through the type I receptor, which is the activin-like receptor (ALK, 4, 5, 7), serine/threonine kinase receptors. The intracellular domains recruit and phosphorylate the receptor-regulated Smad (R-Smad) proteins, such as Smad 1, 2, 3, and 5, in homo or hetero dimers. Smad4 is a common mediator of Smad (Co-Smad), which, in interaction with R-Smad proteins, functions as a transcription factor; consequently, this leads to nuclear translocation and gene expression. The negative regulations are mediated by the inhibitory Smad (I-Smad) proteins, such as Smad 6 and 7 or Smurf 1/2 proteins. The non-canonical pathways are represented by the mitogen-activated protein kinase (MAPK), TRAF6,

and TAK1. In Figure 4, the intracellular activation routes mediated by GDF11 are presented (Gerardo-Ramirez et al., 2023; Simoni-Nieves et al., 2019).

2. 10. 2. The biology of GDF11 in the context of cancer.

The effects of GDF11 have been controversial over the years, even in cancer. The updated reports show the antitumoral effects of GDF11 in many cancers, such as breast, pancreatic, and HCC (Bajikar et al., 2017; Gerardo-Ramírez et al., 2019; Y. Liu et al., 2018). Strikingly, this is now tested in leukemias (SÁNCHEZ-RODRÍGUEZ et al., 2025)

GDF11 can decrease the aggressive phenotype in different HCC cell lines through a mechanism mediated by Smad 2 and 3. Furthermore, GDF11 reduces parameters like proliferation, invasion, and migration by promoting the mesenchymal to epithelial transition (MET) process (Gerardo-Ramírez et al., 2019). Additionally, GDF11 decreases cholesterol and triglyceride levels in human HCC cell lines, suggesting it is a regulator of lipid metabolism (Hernandez et al., 2021; Lu et al., 2019). Given the implications of GDF11 in cancer, this could represent an effective therapy in many types of tumors, including HCC.

3. JUSTIFICATION.

As mentioned before, cancer is a lethal disease according to the WHO (<https://www.who.int/news-room/fact-sheets/detail/cancer>). Malignant tumors, such as liver cancer, have been ranked sixth and third in terms of incidence and mortality, respectively, worldwide. According to the latest update, in 2022, liver cancer accounted for 0.87 million new cases and 0.76 million deaths, and is projected to increase significantly by 2050 to 1.52 million new cases (Chan et al., 2025). On the other hand, the increase in cancer-related deaths in

Mexico is putting it in third place and is a great problem for this Latin American country. Heart problems and diabetes mellitus also represent great problems in first and second place, respectively. According to a recent Mexican press release, in 2023, 799,869 new deaths occurred, of which 91,562 (11.4 %) were new cancer-related deaths, 52.4% in women and 47.6% in men. Strikingly, liver and intrahepatic bile duct cancers are among the four most frequent cancer-related deaths in this country, but could vary according to age (INEGI, 2025). Additionally, deaths associated with liver diseases represent the fourth place (INEGI 2025), which could be associated with the development of the other main pathologies, due to the high versatility of the liver.

Moreover, the ingestion of a lipid/cholesterol-rich and fructose-rich diet, independent of other risk factors, is associated with the development of the most aggressive forms of liver cancer, including HCC or MASH-HCC (Chávez-Rodríguez et al., 2025; Enríquez-Cortina et al., 2017; Simoni-Nieves et al., 2021). Moreover, the increase in TAMs with M2-like polarization recruitment makes it more challenging to treat due to the friendly relationship with transformed cells (Escobedo-Calvario et al., 2022). Furthermore, these MASH-HCC limit anti-tumor surveillance using immunotherapy, which, in short, leads to an increase in tumor aggressiveness (Pfister et al., 2021). Therefore, the design of effective therapies against HCC is the main aim in the recent world.

Finally, the use of molecules to modulate tumor aggressiveness and TME cells, such as TAMs, could significantly mitigate this pathology and increase patient survival. GDF11 has been associated with a good response against HCC cancer cells (Gerardo-Ramírez et al., 2019; Hernandez et al., 2021) and has recently been linked to a significant effect in leukemia, specifically in undifferentiated leukocytes (SÁNCHEZ-RODRÍGUEZ et al., 2025). Using this

information regarding GDF11 could represent a significant step toward treating HCC tumors from two flanks, the tumor core, and TAMs; however, further investment and initial investigation in *in vitro* experimental models are needed.

4. RESEARCH QUESTION.

Does GDF11 treatment reduce the pro-tumoral activity of M2-like macrophages in an HCC *in vitro* model?

5. HYPOTHESIS.

According to the evidence, GDF11 reduces aggressive behavior in HCC-derived cells or leukemia cells, as previously reported. Currently, we hypothesize that GDF11 can also reprogram the tumor immune microenvironment, specifically targeting pro-tumoral macrophages with M2-like polarity, thereby reducing their malignant cooperation with transformed cells.

6. AIM.

To evaluate the anti-tumoral response of GDF11 on M2-like THP-1 differentiated macrophages in the interaction with HCC cell lines.

7. SPECIFIC AIMS.

- To standardize the antitumoral effects of GDF11 on the THP-1 differentiated macrophage cell line.
- To evaluate the M2-like macrophage polarity in response to GDF11.

- To characterize the metabolic and redox status induced by GDF11 on THP-1 polarized macrophages.
- To describe the interaction of GDF11-treated M2-like THP-1 macrophage on the HCC (Huh7) cell line.
- To analyze the secretory cytokines mediated in GDF11-treated macrophages.

8. MATERIAL AND METHODS.

Human recombinant growth differentiation factor 11 (hGDF11) (Peprotech, Inc., 120-11, Rocky Hill, NJ, USA) was used for the *in vitro* study. For THP-1 monocyte-macrophage differentiation, we used Phorbol-12-myristate-13-acetate, PMA (Sigma-Aldrich, P1585, Saint Louis, MO, USA); for macrophage activation, we applied lipopolysaccharides, LPS (Sigma-Aldrich, L2630), interferon-gamma, IFN- γ , IL-4, IL-13, and TGF- β (Peprotech 300-02, 200-04, and 200-13) according to required polarity.

8. 1. Management of cell cultures.

For this research, we used THP-1 monocytes and Huh7 cells (HCC cell line), which were obtained from the American Type Culture Collection (ATCC, TIB-202 and CRL-10317, Manassas, VA, USA). Both cell lines were cultured in RPMI 1640 (Gibco, 31800-014, New York, USA) and Williams' Media E (Sigma-Aldrich, 4125-1), respectively. For the complete media for all cultures, we blended with 10% fetal bovine serum (FBS, Hy-Clone, SH30088.03, Logan, UT, USA), 100 U/mL ampicillin, and 100 μ g/mL 8 streptomycin (Gibco, 15240-062). Cells were maintained at 37°C in a 5% CO₂ and 90% humidity atmosphere using a CO₂ incubator (Thermo Fisher, 51030902, Massachusetts, USA). All cultures were maintained in sterile conditions and mycoplasma-free.

8. 2. Cell viability and proliferation assays

For each well, we used 100 μ L and seeded 5×10^3 or 1×10^4 for Huh7 and THP-1 cell lines, respectively, in 96-well plates (Corning Inc., 3599, Massachusetts, USA). Cell Counting Kit-8 (CCK-8, Dojindo Lab, CK04, Kumamoto, Japan) or crystal violet (Sigma-Aldrich, 42555)

addressed cell viability and proliferation, following the manufacturer's instructions. Cytarabine (AraC, Sigma-Aldrich, C1768) 10 μ M was used as a negative control for proliferation, and dimethyl sulfoxide (DMSO, Sigma-Aldrich, 472301) for viability. FBS was used as a positive control for proliferation. We quantified the final absorbance at 450 nm every 24 h and compared the mean with the curve in every assay for proliferation, serving as a reference for cell numbers at every time.

8. 3. Macrophage polarization protocol.

For macrophage activation and specific polarization, we followed the reported protocol (Dong et al., 2019) with some modifications. We used 1×10^6 THP-1 monocytes plated in a 60 mm dish (Corning, 430166), which were differentiated into inactivated macrophages (M0) using PMA (200 ng/mL) for 24 h in 2 mL as a final volume. After this time, we wash three times using PBS to discard the remnant PMA in the culture. To induce M1-like macrophage polarity acquisition, IFN- γ (20 ng/mL) or LPS (100 ng/mL) was applied for another 24 h; additionally, for M2-like activation, we used the anti-inflammatory cytokines, such as IL-4 (20 ng/mL) and IL-13 (20 ng/mL) for another 72 h in 2 mL as a final volume. The last treatments were co-treated using GDF11 (50 ng/ml), three doses every 24 h, without exchanging media to simulate tumor conditions such as acidification. Finally, cells were used for subsequent experiments, conditioned media (CM) were collected, and stored at -20 °C for physiological assays. Graphical experimental design is shown in Figure 5. Positive controls of M2-like macrophages used CM derived from 1×10^6 Huh7 cells and applied on M0 (1:1 dilution) for 24 and 72 h to observe the increment of CD206 marker.

8. 4. GDF11 post-treatment protocol.

M2-like macrophages were treated for an additional 72 h in the presence of IL-4/13 (Post M2-like) in combination with GDF11, using new media, three doses, each added every 24 h (denoted as Post M2-like + GDF11). We used the cells for the subsequent experiments and recovered CM as mentioned above, also described in Figure 5.

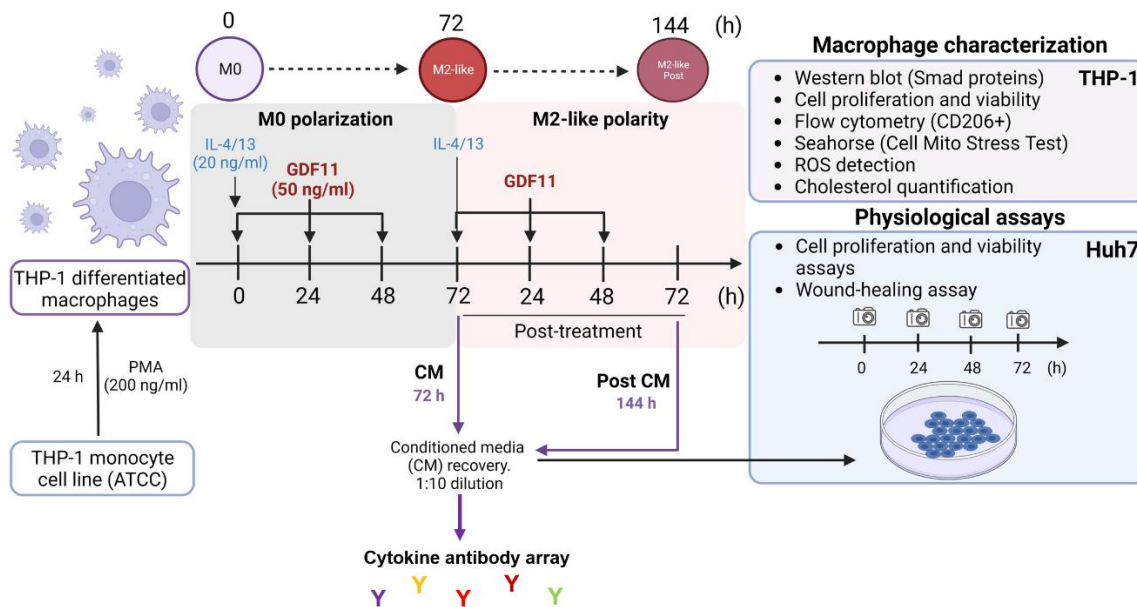


Figure 5. General experimental design of the study. The image summarizes the protocol for macrophage differentiation and polarization; moreover, it describes physiological experiments using HCC cells. Image created using Biorender.com.

8. 5. Macrophage detachment and preparation.

After treatments, we followed the protocol to detach cells using a solution PBS-EDTA (5mM) for 1 h in slow agitation on ice, which is a better form to maintain extracellular proteins and cell viability in macrophages (Chen et al., 2015). We recovered the cells in a microtube, added complete media, and then centrifuged them at 2800 rpm for 5 min at 4°C. The cells were washed

three times using PBS-FBS (2%). To fix cells, we used 500 μ L of PBS-formaldehyde (4%) for 5 min on ice. An important step was to discard the formaldehyde, centrifuging at 2800 rpm for 5 min at 4°C, and finally, we added PBS. Later, we used PBS-BSA 2% as a blockade solution for 30 min at RT, and we discarded it using centrifugation mentioned above. Finally, we applied PBS-BSA (0.5%) to the cells and prepared for the next experiment.

8. 6. Flow cytometry analysis.

To identify M2-like macrophage subsets, the CD206 antibody was applied to the cells overnight at 4°C in agitation. The secondary antibody was applied for two h, at RT in the dark and agitation. The specifications of each antibody used in the study are listed in Table 1. Flow cytometry (FCM) analysis was performed by analyzing 1×10^4 events in duplicate. We applied PBS-heparin to avoid cell agglutination. The study utilized a BD FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA), and the data obtained were analyzed using FlowJo v10.8 Software (BD Biosciences).

Table 1. Antibodies used in the study

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)	0.6-1 μ g FCM	Mouse mAb	Santa Cruz Biotechnology, sc-58986
GDF11	30 ng WH	Mouse mAb	Santa Cruz Biotechnology, sc-81952
β-actin	1:10000 WB	Rabbit mAb	Millipore-Sigma, A3854
Alexa Fluor 488	1:500 FCM	Mouse mAb	Thermo Fisher, A32723

8. 7. Protein isolation and quantification.

We seeded 1×10^6 cells in a 60 mm dish according to the protocol for macrophages. The cells were detached with PBS using a scraper and then centrifuged at 18,000 rpm for 15 seconds. 50-100 μ L of M-PER lysis buffer (Thermo Fisher Scientific, 78501, Massachusetts, USA), which contained protease inhibitors (Complete, Sigma-Aldrich, 11836153001) and phosphatase inhibitors (PhosSTOP, Sigma-Aldrich, 4906845001)

was used in the pellet, shaken, and stored at -20°C . The protein extraction was performed by strongly shaking (vortexing) the pellets 3 times for 30 seconds, and centrifuging at $13,000 \times \text{rpm}$ for 15 min at 4°C . The supernatant was collected and stored at 20°C . Protein quantification was performed using the bicinchoninic acid (BCA) kit (Pierce, BCA Protein Assay Kit, Thermo Fisher

Scientific, 23225) and following the manufacturer's instructions. The mix was incubated for 30 min at 37°C and then quantified at 595 nm. We used 30-100 micrograms of protein for THP-1 macrophages and Huh7 cells in the Western blot assays.

8. 8. Western blotting.

The Western blots were performed using the SDS-PAGE protocol. All samples were treated with β -mercaptoethanol, heated for 7 min at 95°C, and then loaded in a 10% gel. All the electrophoreses were at 120 V at RT. Gel proteins were transferred to a PVDF membrane (Sigma-Aldrich, St. Louis, MO, USA) at 120 V for 2h at 4°C using dry transfer. The membranes were blocked using fat-free milk (1%) dissolved in TBS-Tween for 1h. Primary and secondary antibodies and specific dilutions are listed in Table 1. Blot images were visualized using a ChemiDoc Imaging System (Bio-Rad, Hercules, California, USA), and blot densitometry was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). All data were normalized using β -actin (Sigma Aldrich, A3854) as a loading control.

8. 9. Real-time metabolism determination.

For real-time metabolism, the oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) were standardized using four cell densities (25, 50, 100, and 150×10^3) with THP-1 differentiated macrophages and five carbonyl cyanide-4- (trifluoromethoxy)phenylhydrazone (FCCP) concentrations (0.125, 0.25, 0.5, 1, and 2 μ M) using the Seahorse XFe24 Flux Analyzer and XF Cell Energy Phenotype Test (Agilent Technologies, 103325-100, Santa Clara, California) according to the manufacturer's instructions. All cultures were plated in an XFe24-well plate

(Agilent Technologies, 100777-004) and supplemented with XF RPMI (Agilent Technologies, 103576-503) phenol red-free.

For the XF Cell Mito Stress Test Kit (Agilent Technologies, 103015-100), we followed the manufacturer's instructions and reseeded THP-1-treated macrophages in the XFe-well plate 24 hours before initiating the assays. We used 5×10^4 per well previously characterized. The XF RPMI media was supplemented with 1 mM pyruvate (Sigma-Aldrich, S8636), 2 mM glutamine (Sigma-Aldrich, G8540), and 10 mM glucose (Sigma-Aldrich, G7021), adjusted to a pH of 7.4, and filtered with a 0.22 μ m filter (Corning, 431219) to maintain sterile conditions. Later, we removed the treatment media to apply XF RPMI carefully with 3 washes according to the original protocol to avoid mechanical stress. We used 500 μ L as the final volume in each well and incubated the cultures at 37°C CO₂-free for 1 h. The assay followed the sequential injections of 1.5 μ M oligomycin A (Sigma-Aldrich, 75351), 1 μ M FCCP (Sigma-Aldrich, C2920), and 0.5 μ M antimycin A (Sigma-Aldrich, A8674) plus Rotenone (Sigma-Aldrich, R8875). The inhibitors were prepared independently of the Kit. Finally, at the assay's end, we discarded the entire XF medium and added 30 μ L of lysis buffer (Sigma-Aldrich, R0278) for subsequent freezing (-20 °C). We use a 200 μ L pipette tip to scratch the well and obtain the protein. Therefore, the OCR and ECAR measurements were normalized with total protein according to our BCA protein quantification protocol.

8. 10. ROS detection.

ROS detection was performed using dihydroethidium (DHE, Thermo Fisher Scientific, D11347) at a final concentration of 5 μ M. THP-1-treated macrophages were seeded in a 35 mm dish (Corning, 430165), performing a density math for this format. DHE was diluted in PBS 1 $\times$ (Sigma

Aldrich, D5773) and applied on THP-1 differentiated macrophages for 15 min at 37 °C in the dark; images were captured using the Cy3 filter of Axio Vert.A1 microscope (Carl Zeiss, Jena, Germany); finally, images were analyzed (200×) every 24 h, using ImageJ software (National Institutes of Health, NIH, Bethesda, MD, USA). We applied rotenone (2 µM) and antimycin (5 µM) as positive controls. For the indirect phagocytosis assay detection through ROS, macrophages were treated with GDF11 24 hours prior, and the latex beads (Sigma-Aldrich, LB6) were used at a 1:100 cell-bead ratio for 90 minutes.

8. 11. Cholesterol quantification

Cholesterol quantification was done according to previously reported methods (Simoni-Nieves et al., 2021). Briefly, approximately 1×10^6 cells were saponified with alcoholic KOH solution. Hexane (Sigma Aldrich, 34859-2) and distilled water were added and shaken to ensure complete blending. The superior layer with hexane was recovered in new microtubes and evaporated (approximately 1 hour) under a vacuum (Speed Vac, Savant, Cranbury, NJ, USA). Total cholesterol was measured using O-phthalaldehyde (Sigma Aldrich, P1378) dissolved with acetic acid (0.5 mg/mL, MERK, 9508-05). Final reaction was measured at an absorbance of 550 nm. The cholesterol concentration was compared with a cholesterol curve and normalized with total protein in each sample according to our protein quantification method. We used one liver sample from high-cholesterol diet-fed C57BL/6 male mice as a positive control, donated by the group of Arturo Simoni-Nieves.

8. 12. Migration as a wound-healing assay

To assess the migratory process, we used wound-healing inserts (Ibidi, 81176, Gräfelfing, Germany) and seeded 3×10^4 Huh7 cells per well. The cells were incubated overnight. The next day, the insert was removed, and the dishes were washed gently with PBS 1× to remove the debris and waste. Subsequently, Williams' Medium E media was added in the presence or absence of conditioned media (CM) derived from treated macrophages in a 2 mL final volume. CM media dilutions were 1:1, 1:2, 1:3, 1:4, 1:8, and 1:10, into fresh Williams' media (CM: Williams' media, respectively). In all cases, we previously centrifuged to discard cellular debris. The healing closure was monitored, and images (100×) were taken every 24 h up to 72 h using an Axio Vert.A1 microscope. The wound area (μm^2) and migration capacity (%) were analyzed using ImageJ software. The anti-GDF11 (Santa Cruz, sc-81952) antibody (1.62621×10^{12} molecules) was mixed in CM derived from treated macrophages, previously filtered, and stirred overnight at 4°C before use. More specifications of anti-GDF11 are in Table 1.

8. 13. Cytokine detection array.

Cytokine detection (42 targets) was performed using the Human Cytokine Antibody Array Membrane (Abcam, ab133997) according to the manufacturer's instructions. For this purpose, we used 1 mL of undiluted CM derived from treated and untreated THP-1 macrophages. Blot images were revealed using the ChemiDoc™ Imaging System (Bio-Rad), and the dot blot analysis was performed using ImageJ software (NIH). The array data normalization of mean pixel density was performed using the mathematical formula $X(Ny) = X(y) * P1/P(y)$, according to the specification of the kit.

8. 14. *Statistical analysis.*

GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA) was used for data statistics and analysis on a Windows platform. The entire data is presented as the $\pm$ standard error of the mean ($\pm$ SEM). The analysis of variance (ANOVA) test was used to compare mean values among groups. A p-value ≤ 0.05 was considered statistically significant.

9. RESULTS

9.1. GDF11 activates the Smad signaling pathway in THP-1 differentiated macrophages without cytotoxic effects.

Firstly, to evaluate the possible effects of GDF11, we evaluated the activation of the canonical signaling pathway in THP-1 differentiated macrophages (M0). We applied one dose of GDF11 at early times (0-60 min) and assessed the specific phosphorylation of Smad 2/3 proteins by Western blot (**Figure 6**). The activation was observed at 5 min, with both proteins being stronger in Smad2 than in Smad3 (**Figure 6 A-C**). Also, we evaluated the content of the primary canonical inhibitor of the route, Smurf1 (**Figure 6A and D**), observing no significant changes.

Our group previously published that GDF11 exerts its biological function over a long period of stimulus (Gerardo-Ramírez et al., 2019); this was a fact that led us to analyze whether GDF11 compromises cell viability and proliferation in our cell line. The data were reflected in the number of cells at 24, 48, and 72 h, with GDF11 treatment administered every 24 h, as depicted in the experimental design (**Figure 5**). Furthermore, we did not detect any changes in cell viability (**Figure 6E**) or proliferation (**Figure 6F**). This result confirms the absence of significant changes in cellular morphology (**Figure 6G**). Therefore, our data suggest that GDF11 does not induce any cytotoxic response in the THP1-differentiated macrophage cell line used in the research.

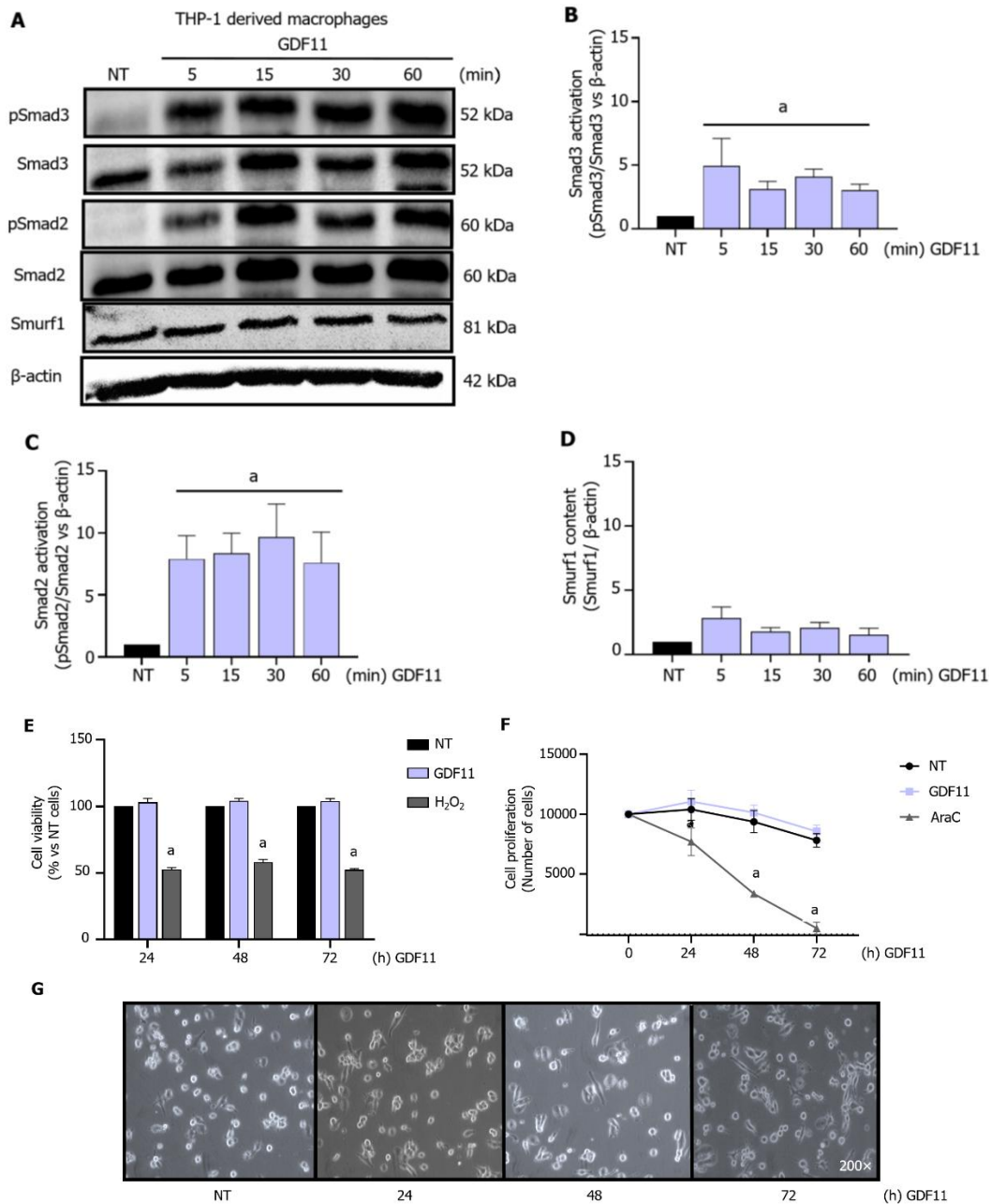


Figure 6. GDF11 induces Smad protein activation without cytotoxicity in macrophages. A: Representative blots; and B-D: densitometry analysis. β-actin was used as a loading control; E: Cell viability. H₂O₂ was used as a negative control; F: Proliferation by number of cells. AraC was our negative control. Each column or point represents the mean ± SEM of at least three independent experiments carried out in triplicate. ^a*P* ≤ 0.05 vs not treated cells (NT); G: Macrophage morphologies at 200×, original magnification; each image is representative of at least three independent experiments.

9.2. M2-like macrophage standardization.

Once we clarified that GDF11 activates expected routes in our cultures, we continued to standardize our protocol for macrophage polarization. According to the protocol description, we differentiated the THP-1 monocyte into inactivated (M0) macrophages using PMA for 24 h. After that, we treated M0 cells with IL-4 in combination with IL-13 for 24 and 72 h to obtain an M2-like activation; LPS or INF- γ was used to induce M1-like subsets. We called it “M2-like” because we used a combination of GDF11 in some cultures, and to homogenize our polarized terms. The results confirmed the M2-like polarization acquisition using an anti-inflammatory stimulus according to FCM analysis of CD206-positive cells (CD206⁺) (**Figures 7, 2A-C**). Furthermore, we observed an extended or bipolar morphology in IL-4/13-treated macrophages at 72 h, features of these macrophages *in vitro*. Unlike the M2-like, the M1-like phenotype possesses a single pole (**Figure 7D**).

On the other hand, we decided to compare the IL-4/13-treated macrophages with CM-treated macrophages (CM from Huh7 at a 1:1 dilution) as a positive control of our *in vitro* model, observing the CD206 content and positive cells by FCM. Cancer cells produce a rich cocktail of mediators that can simulate TME signals. In both stimuli, we observed an increase in CD206⁺ cells; however, our first protocol undoubtedly represents a purer model to replicate, as it excludes additional factors that could bias our study (**Figure 8 A-C**).

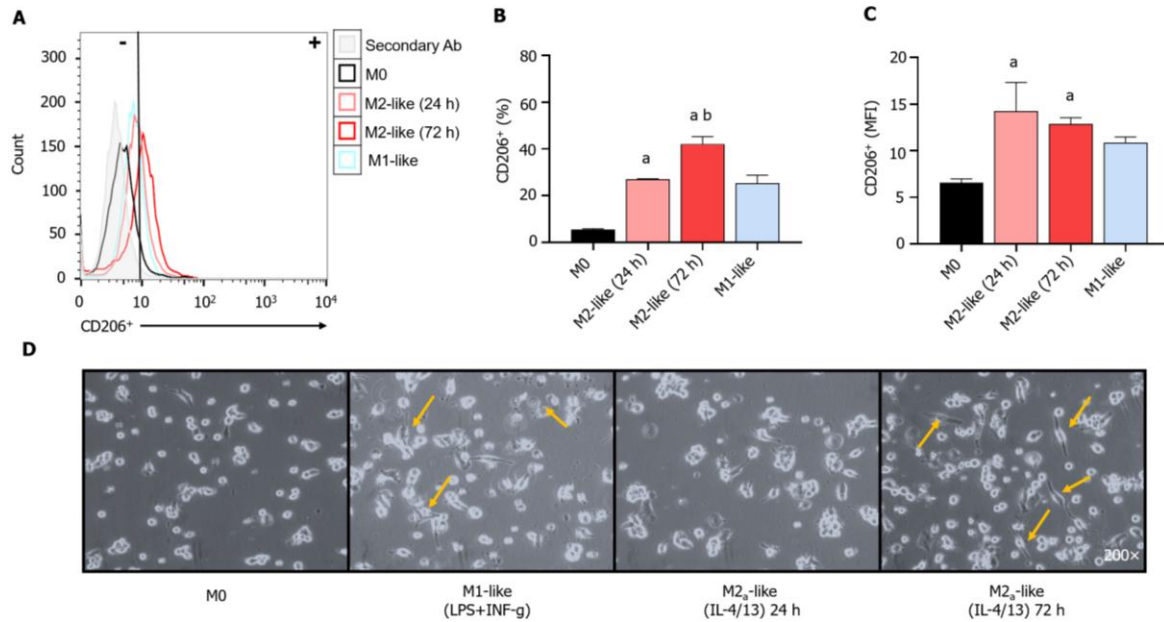


Figure 7. The M2-like macrophage acquisition. A: Fluorescence histogram of the CD206 marker by FCM; B: CD206⁺ cells (%) in IL-4/13-treated macrophages; C: Mean fluorescent intensity (MFI) of the CD206⁺ subset. Each column represents the mean $\pm$ SEM of at least three independent experiments. ^a $P \leq 0.05$ vs. M0, ^b $P \leq 0.05$ vs. M2-like (24 h). M1-like macrophages (24 h) were used as a control; D: Macrophage morphology under the stimuli, 200 $\times$ original magnification. The orange arrow shows specific morphology. Images are representative of at least three independent experiments.

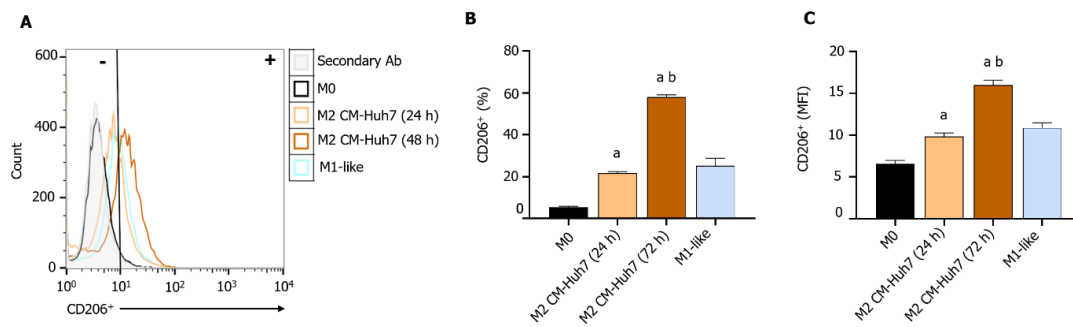


Figure 8. The M2-like macrophage acquisition by CM from Huh7. A: Fluorescence histogram of the CD206 marker by FCM; B: CD206⁺ cells (%) in CM-treated macrophages; and C: Median Fluorescence Intensity (MFI) of CD206⁺ subset. Each column represents the mean $\pm$ SEM of at least three independent experiments. ^a $P \leq 0.01$ vs M0, ^b $P \leq 0.05$ vs M2 CM-Huh7 (24 h). M1-like macrophages (24 h) were used as a control.

9.3. GDF11 mitigates the CD206⁺ subsets from M2-like macrophages

Before standardizing this protocol, we first recognized that the absence of an anti-inflammatory effect after 72 h can reduce CD206 content (data not shown). Therefore, the exchange media and the new addition of IL-4/13 treatment induce the maintenance and increment of this receptor. The new stimulus using GDF11 as a co-treatment was considered competence-like.

After macrophage activation and standardization, we evaluated the CD206⁺ subsets in combination with GDF11 by FCM according to the protocol. We observed that GDF11 reduced the percentage of CD206⁺ subsets in M2-like macrophages to 72 h (early response) from ~40% to ~25%, suggesting the possible tampering or re-polarization of these cells (**Figure 9A-C**).

The next step was to apply an additional 72 h of treatment (Post M2-like; extended response), new doses of IL-4/13 and GDF11. This condition showed that GDF11 also reduced the CD206⁺ macrophage subsets (%) from ~90% to ~30%, indicating a reduction in the pro-tumoral phenotype (**Figure 9D-F**).

CD206 protein, a surface or membrane mannose receptor, is a marker of aggressiveness in cancer cells and TAMs. Therefore, this result supports the notion that GDF11 affects M2-like polarization and, consequently, the pro-tumoral properties. At this point, our data suggest that GDF11 displays a re-educating or neutralizing effect in M2-like macrophage subsets.

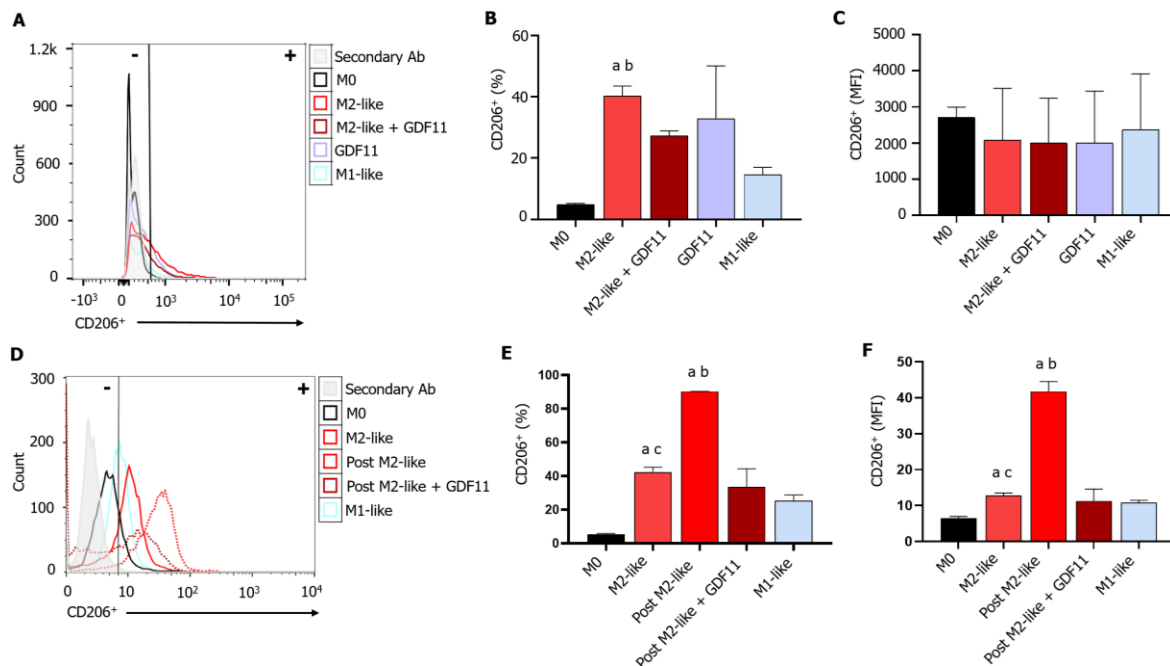


Figure 9. GDF11 decreases CD206⁺ in M2-like macrophages. A: Fluorescence histogram of the CD206 marker by FCM at 72 h; B: CD206⁺ cells (%) at 72 h; C: Median Fluorescence Intensity (MFI) of the CD206⁺ subset at 72 h; D: Fluorescence histogram of the CD206 marker by FCM at 144 h; E: CD206⁺ cells (%) at 144 h; F: MFI of the CD206⁺ subset at 144 h. Each column represents the mean $\pm$ SEM of at least three independent experiments. ^a $P \leq 0.01$ vs M0, ^b $P \leq 0.05$ vs. Post M2-like + GDF11, and ^c $P \leq 0.001$ vs. Post M2-like.

9.4. Characterization of the number of cells and FCCP concentration for the metabolic assay.

Following the observed changes in macrophage polarization and the loss of the CD206 marker in M2-like macrophage subsets using GDF11, we focus on studying the immunometabolism, which is strongly linked to specific responses. First, we standardized the number of cells seeded to 25,000, 50,000, 100,000, and 150,000, and evaluated the density under the bright microscopy (**Figure 10A**). In the second experiment, we evaluated the FCCP concentration at 0.125, 0.25, 0.5, 1, and 2 μ M. In both essays, we determine the levels of OCR and ECAR using the XF Cell energy phenotype test according to protocol. We conclude that 50,000 cells per well was the

ideal density (**Figure 10B-C**) and 1 μM of FCCP promotes major effects in the metabolic context (**Figure 10D-E**).

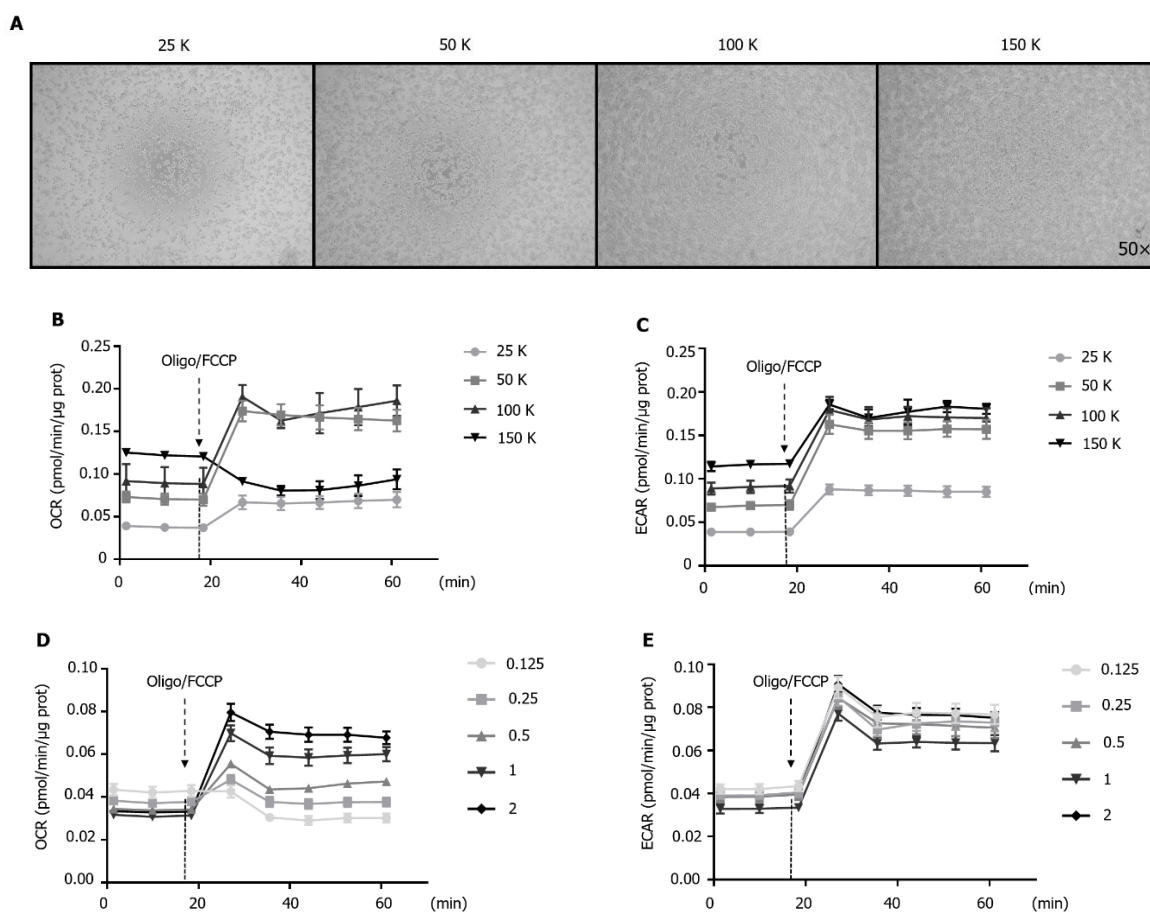


Figure 10. Number of cells and FCCP standardization for metabolic assays. A: Macrophage culture densities under bright microscopy, 50 $\times$ original magnification. Images are representative of at least three independent experiments. The XF Energy phenotype test was performed to evaluate the number of cells, as measured by B: OCR and C: ECAR landscapes, and the FCC concentration measured by D: OCR and E: ECAR landscapes. Each point represents the mean $\pm$ SEM of at least three independent experiments with $P \leq 0.05$.

9.5. M2-like macrophages present differences in bioenergetics.

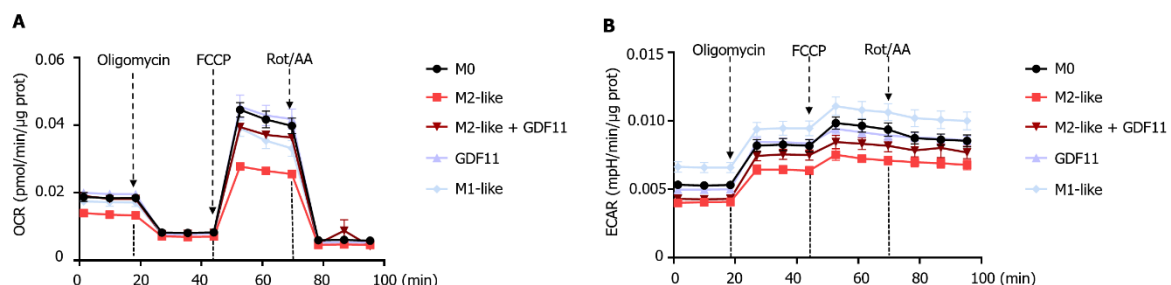
Our protocol for these *in vitro* macrophages showed that M0 macrophages (black line) had a higher OCR value in normal and stress conditions (FCCP) than M2-like macrophages (red line)

in this experimental model. M2-like macrophages exhibit a decrease in mitochondrial respiration, as reflected in the OCR levels depicted in the metabolic profile or landscape (**Figure 11A**). Interestingly, the GDF11 treatment in M2-like macrophages (dark red) appears to be resetting the parameters observed in M0, which aligns but differs from our previous work in HCC cell lines (Hernandez et al., 2021). Notably, GDF11 treatment maintained the OCR levels, suggesting no relevant role in bioenergetics when it is used alone (purple line).

ECAR levels were also quantified by Seahorse, showing that LPS promotes media acidification and suggesting an alternative metabolic route for anti-inflammatory macrophages, which are characterized by low ECAR levels (**Figure 11B**).

Maximal respiration was the most prominent parameter affected due to the exacerbated respiration of GDF11-treated and M0 macrophages (**Figure 11C**). Basal respiration (**Figure 11D**), ATP production (**Figure 11E**), and spare respiratory capacity (**Figure 11F**) exhibited similar behavior, with no changes observed in non-mitochondrial OCR in all groups (**Figure 11G**), indicating specific alterations within this organelle.

Besides, we showed the phenotypic status of treated macrophages in the OCR vs. ECAR axis (**Figure 11H**); the M2-like macrophage acquires a more energetic phenotype after GDF11 treatment (M2-like + GDF11), similar to the effects of GDF11 alone treatment or the M1-like macrophage, suggesting a rewiring in energy production and probably immune responses according to the literature (Angajala et al., 2018).



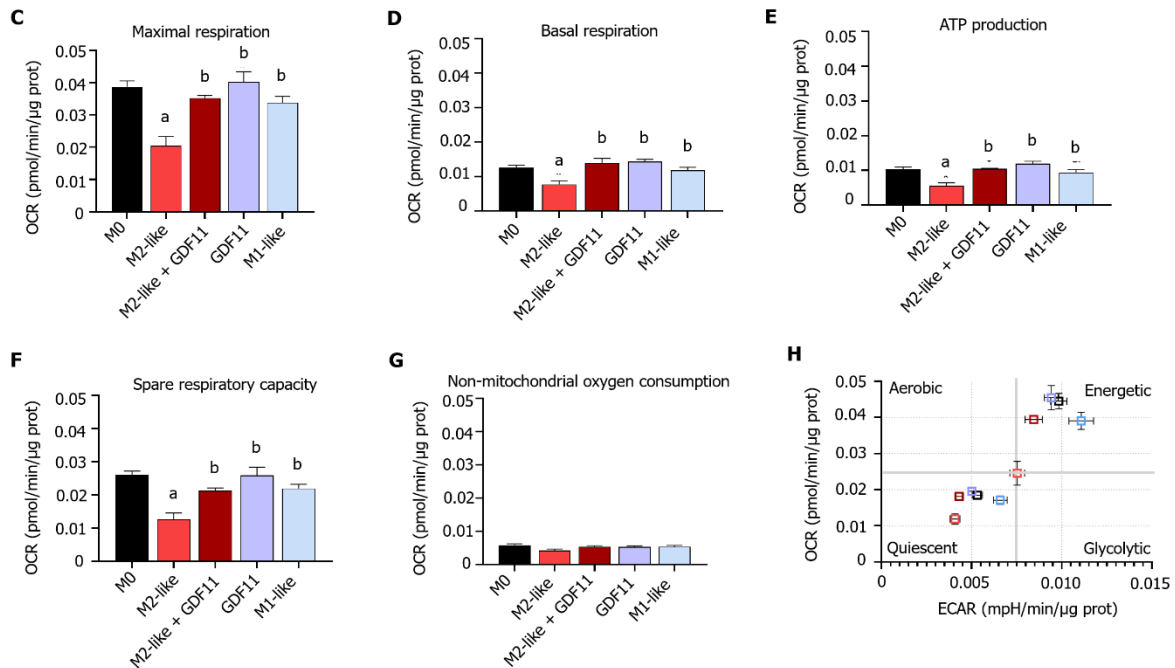


Figure 11. GDF11 promotes bioenergetics rewiring in M2-like cells. The XF Cell Mito Stress Test was performed to evaluate A: OCR metabolic profile, and B: ECAR metabolic profile using Seahorse XF24e flux analyzer. The processed data were depicted as C: Maximal respiration; D: Basal respiration; E: ATP production; F: Spare respiratory capacity; and G: Non-mitochondrial OCR. H: Defective metabolic profile based on relative values of OCR and ECAR before and after metabolic stress induction. Each column represents the mean $\pm$ SEM of at least three independent experiments. ^a $P \leq 0.05$ vs. M0, ^b $P \leq 0.05$ vs. M2-like.

9.6 GDF11 modifies the cholesterol content in THP-1-derived macrophages.

GDF11 decreases cholesterol, as previously reported in HCC cell lines (Hernandez et al., 2021). This fact led us to investigate whether GDF11 possesses similar metabolic features in the lipid context, specifically in macrophages. In our *in vitro* model, we demonstrated that GDF11 reduces cholesterol content in macrophages at 72 h (**Figure 12A**). We compared this reduction using control with atorvastatin, a specific statin, an inhibitor of the 3-hydroxy-3-methyl-glutaryl-CoA

reductase (HMGCR), the rate-limiting enzyme of the mevalonate pathway (**Figure 12B**). This data provides evidence that GDF11 affects macrophages in a lipid-rich context.

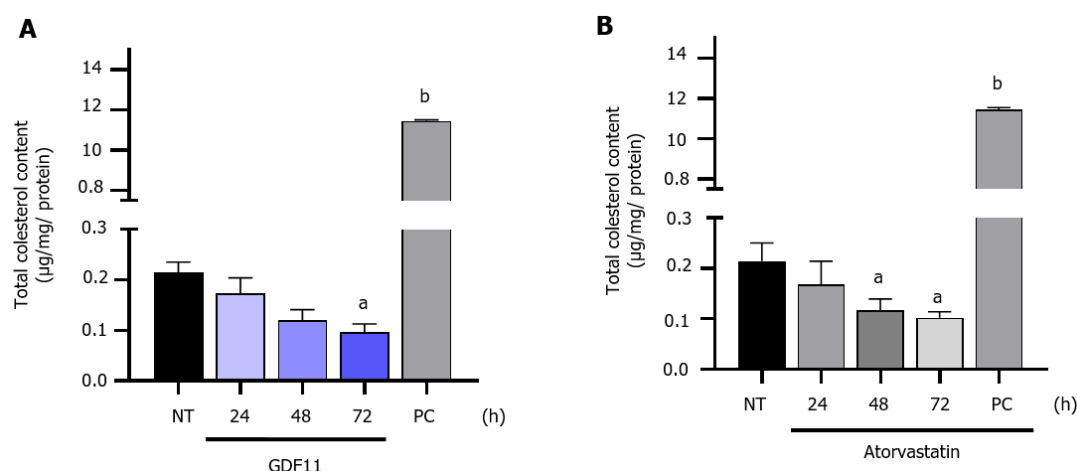


Figure 12. GDF11 decreases cholesterol content in macrophages. A: Total cholesterol content from GDF11-treated macrophages; B: Cholesterol from atorvastatin-treated macrophages. Atorvastatin was used at a 10 μM concentration. PC, positive controls, liver tissue from a mouse fed a high cholesterol diet (2%) for eight months. Each column represents the mean ± SEM of at least three independent experiments in triplicate. ^a $P \leq 0.05$ vs NT, ^b $P \leq 0.0001$ vs NT.

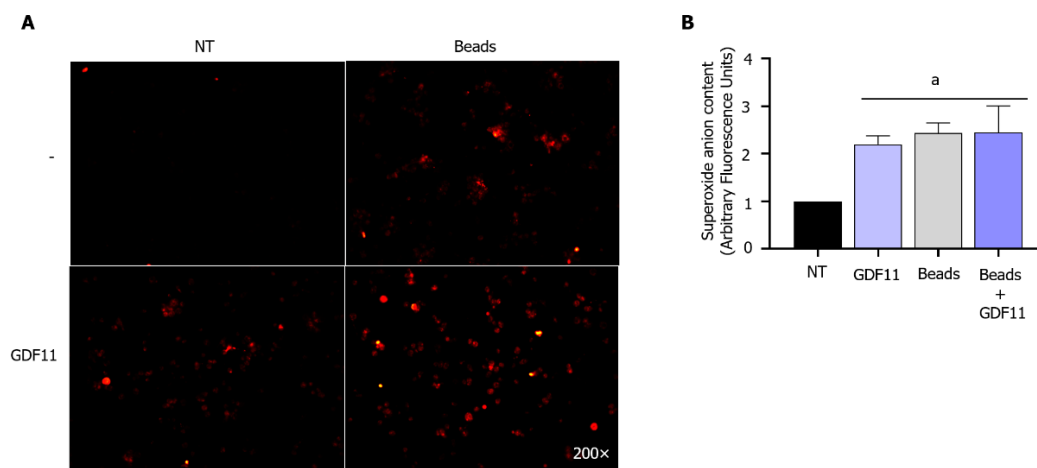
9.7 GDF11 promotes reactive oxygen species production.

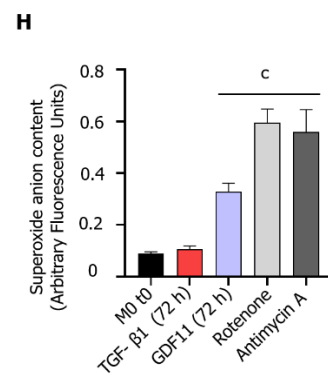
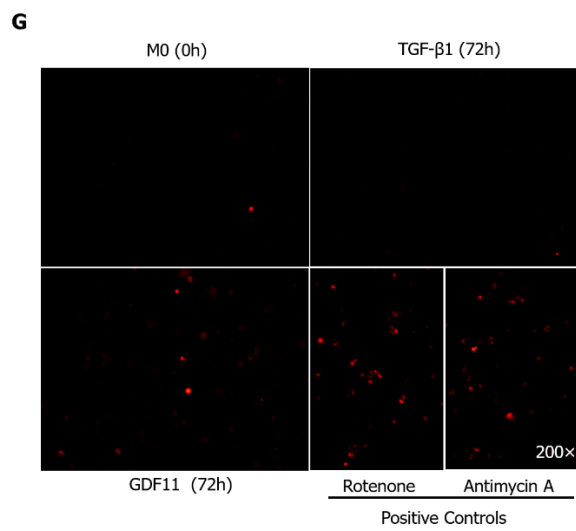
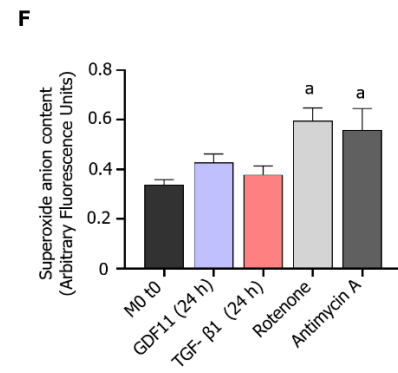
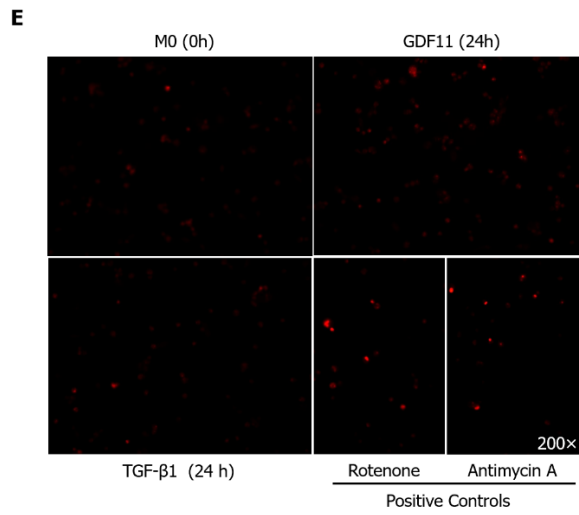
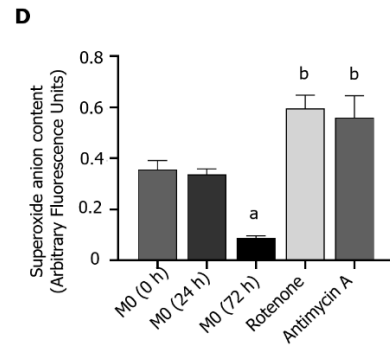
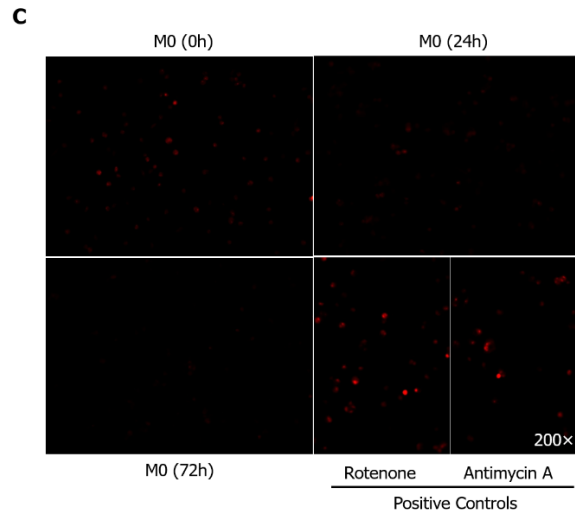
Our data support the hypothesis that GDF11 could alter the phenotype of M2-like macrophages to an antitumoral phenotype. We examine the redox state to gain more information, as the macrophages modified the ROS production in a dependent manner.

First, we tested whether THP-1 differentiated macrophages respond to contact with latex beads to induce phagocytic engulfment. In an indirect manner, we evaluate this process via ROS production, which increased the superoxide anion (O_2^-) content. Strikingly, GDF11 alone (previously treated for 24 h) increased ROS production to similar values observed in bead-

treated macrophages; however, combining both treatments did not induce a significant change compared with the treated groups (**Figure 13A-B**). The result suggests that GDF11 exhibits anti-tumoral and microbicidal features, primarily through high ROS generation, particularly in the phagocytic activity process.

We continue exploring the ROS dynamics after the differentiation process of THP-1 cells. We measured ROS content at 0, 24, and 72 h in M0 cells (**Figure 5C-D**); the ROS levels decreased significantly at 72 h. Later, we moved to figure out the effect of GDF11 on M0 cells; we could not find a difference at 24 h, even in cells treated with TGF β -1 (**Figure 13E-F**), but at 72h, we observed an increment of ROS only in GDF11-treated macrophages, without effect using TGF- β 1 (**Figure 5G-H**). Finally, we compared the ROS production between M2a (IL-4/13) and M2c (TGF- β 1) macrophage subsets. In all GDF11-treated macrophages, the high content of ROS was observed at 72 h, indicating a possible microbicide and anti-tumoral capacity training (**Figures 13 I-K**).





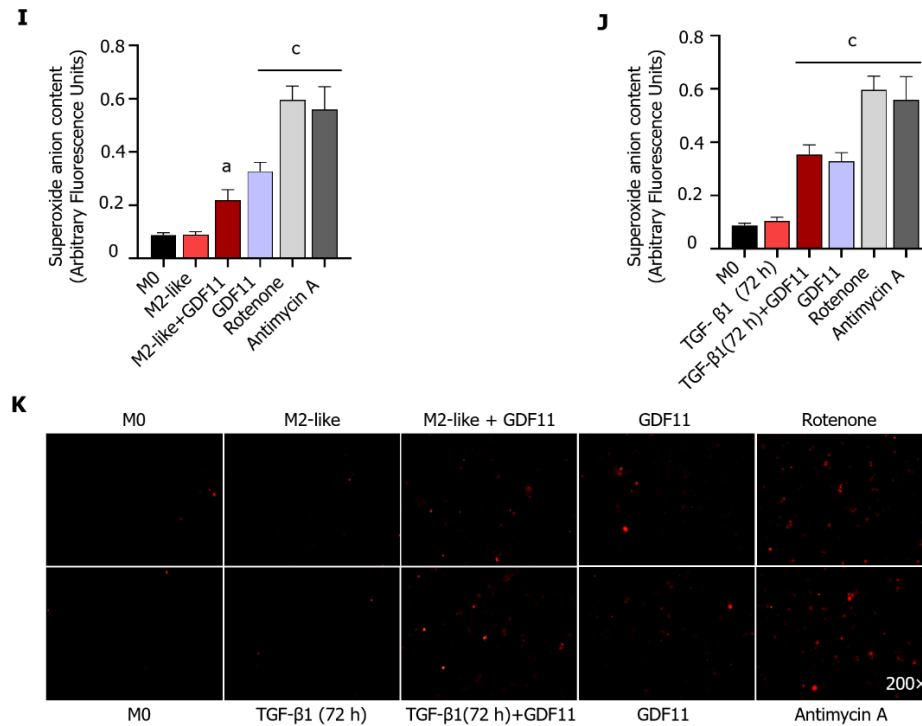


Figure 13. GDF11 increases ROS generation in macrophages. Superoxide anion ($O_2^{\cdot -}$) detection by DHE-derived fluorescence. A, C, E, G, and K: Representative images of DHE-derived fluorescence at 200×, original magnification, using a Carl Zeiss fluorescence microscope; B, D, F, H, I, and J: represent the densitometry analysis. Rotenone (2 μ M) and Antimycin A (5 μ M) were positive controls. Each column represents the mean $\pm$ SEM of at least three independent experiments in triplicate. ^a $P \leq 0.05$ vs NT or M0, ^b $P \leq 0.05$ vs M0, ^c $P \leq 0.01$ vs M0.

9.8 Conditioned media derived from M2-like macrophages increase cell aggressiveness, such as migration, in HCC-derived cells.

As the second part of this research, we focused on characterizing the interactions between GDF11-treated macrophages and cancer cells, such as HCC cell lines. First, we focused on addressing the effects on pro-tumoral properties of M2-like macrophages concerning human Huh7 cell lines by using an indirect co-culture with CM derived from M2-like macrophages.

We assayed cell viability using CM (dilutions 1:3, 1:4, 1:8, and 1:10) at 24, 48, and 72 h. We realized that optimal dilutions were 1:8 and 1:10, observing no cytotoxic effects after 48 h in the

Huh7 cell line (**Figure 14A-C**). The possible reduction in cell viability at 72 h using 1:3 and 1:4 CM dilution may be due to a high concentration of different factors, such as interleukins, growth factors, and acidification.

For further confirmation of aggressivity parameters, we analyzed the migratory capacity of Huh7 cells using a wound healing assay up to 48 h, employing ideal dilutions of 1:8 and 1:10 (**Figure 14D-E**). As expected, the media from M2-like macrophages efficiently closed the wound in a time— and concentration-dependent manner, confirming that M2-like macrophages display pro-tumoral signals that favor the aggressiveness of the HCC cells in our model. Finally, for the subsequent experiments, we decided to use only a 1:10 dilution according to our data.

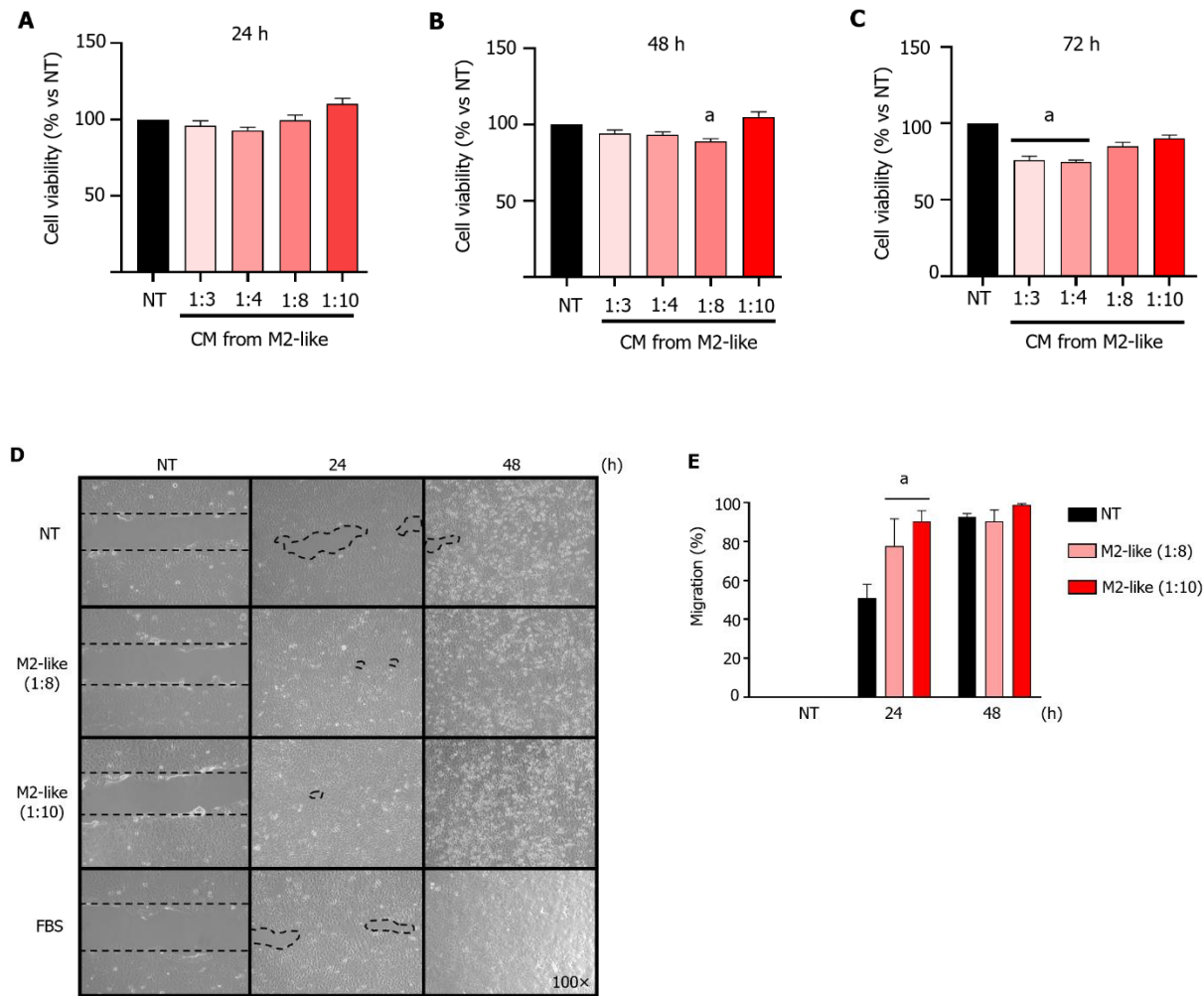


Figure 14. Effects of the conditioned media (CM) derived from M2-like macrophages on Huh7 cells. A-C: Cell viability was assessed using the CCK-8 kit at A: 24 h; B: 48 h; and C: 72 h. CM from M2-like macrophages at different dilutions (1:3, 1:4, 1:8, and 1:10) was used. Each column represents the mean $\pm$ SEM of at least three independent experiments. ^a $P \leq 0.05$ vs NT. D: Cell migration assay determined by a wound healing assay. Representative images of at least three independent experiments, 100 $\times$ of original magnification. E: Plot of the percentage (%) of migration determined by Image J software. Each column represents the mean $\pm$ SEM of at least three independent experiments in triplicate. ^a $P \leq 0.05$ vs NT.

9.9 CM from GDF11-treated macrophages lessens cell aggressiveness in HCC-derived cells.

Following CM standardization on Huh7 cells, we examined the relevance of CM derived from GDF11-treated macrophages on the same cells. We observed that CM from M2-like macrophages treated with GDF11 (M2-like + GDF11 CM) hindered wound closure at 24 h compared to M2-like CM (or not treated with GDF11), showing a reduction or neutralizing effect of TME cells on the aggressiveness of the HCC cell line. We did not observe migratory changes from 48 h to 72 h (**Figure 15A-C**). Besides, we did not find differences in cell proliferation and viability (Data not shown), which indicates that migration is the primary process provided by macrophage secretion in our early time model. Additional control groups and wound area (μm^2) quantification are presented in our extended panel, which shows that CM from GDF11-treated macrophages also delays the wound closure. (**Figure 16A-B**).

CM derived from Post M2-like macrophages and Post M2-like + GDF11 CM exhibits a similar response on Huh7 cells, maintaining these effects until 48 h. We found that the treatment delays wound closure compared with those without the Post GDF11 CM stimulus (**Figure 15D-F**). Additional control groups and wound area (μm^2) quantification are presented in our extended

panels, which show that CM from Post GDF11-treated macrophages also delays the wound closure on cancer cells (**Figure 16C-D**). Besides, we observed a reduction in proliferation and viability using CM derived from all GDF11-treated macrophages (**Figure 15 G-J and 16 E-H**). We must not forget that CM from macrophages could possess a low quantity of remnant rGDF11. To eliminate the potential impact of residual GDF11 molecules in the CM and question the role of macrophage secretion mediators, we treated it with anti-GDF11 and examined migration over 72 hours. Indeed, our result shows that the effect on migration is due to the mediators secreted by the macrophages present in the CM (**Figure 15 K-L**). In the same manner, additional control treatments for this experiment are presented in the extended data panels (**Figure 16 I-J**).

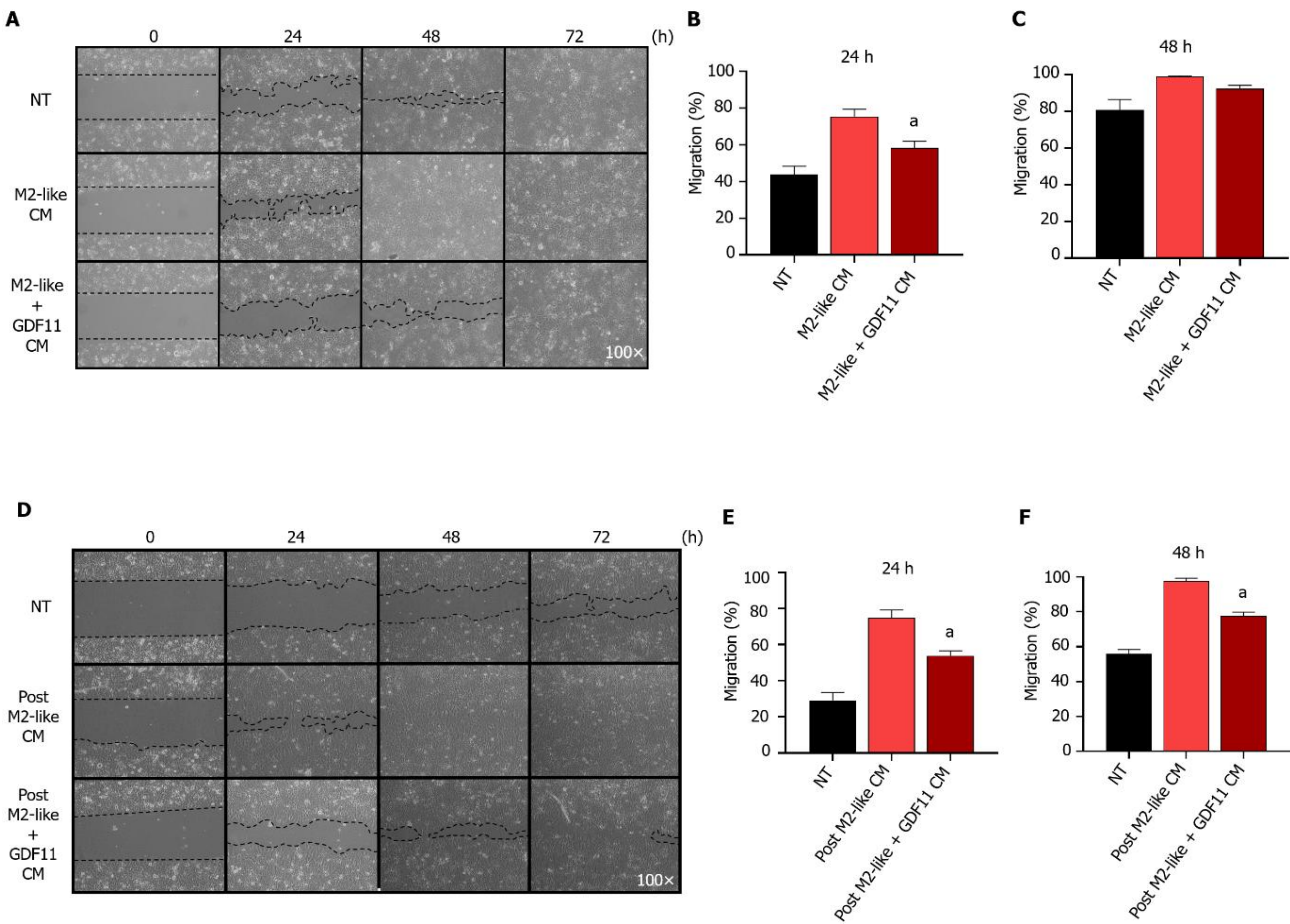
Moreover, to test that the anti-GDF11 antibody effectively blocks soluble rGDF11 in the media or CM, we performed a Western blot of p-Smad3. Anti-GDF11 successfully abolished the activation of this canonical pathway mediated by Smad3 (**Figure 17 A-B**).

As a summary, we have demonstrated and clarified three important points: 1) M2-like macrophages exacerbate tumor aggressiveness, such as proliferation and migration capacity on HCC cells; 2) GDF11 stimuli mitigate pro-tumoral characteristics on M2-like macrophages reflected on cancer cells; and 3) the possible rGDF11 remnants in CM did not affect the results in our models (**Figure 18**).

Finally, to rule out that our wound closures were due to the migration process rather than proliferation, we evidenced the morphologies of the Huh7 cultures at high magnification scale (200×). Our observation shows the extended appearance in Huh7 cultures characteristic of the migratory process. It was important to mention that we excluded the use of specific inhibitors for

proliferation or migration processes, such as AraC, which could reduce cell viability, as we confirmed, and therefore affect the main description in the study **(Figure 19)**.

SHORT VERSION DATA.



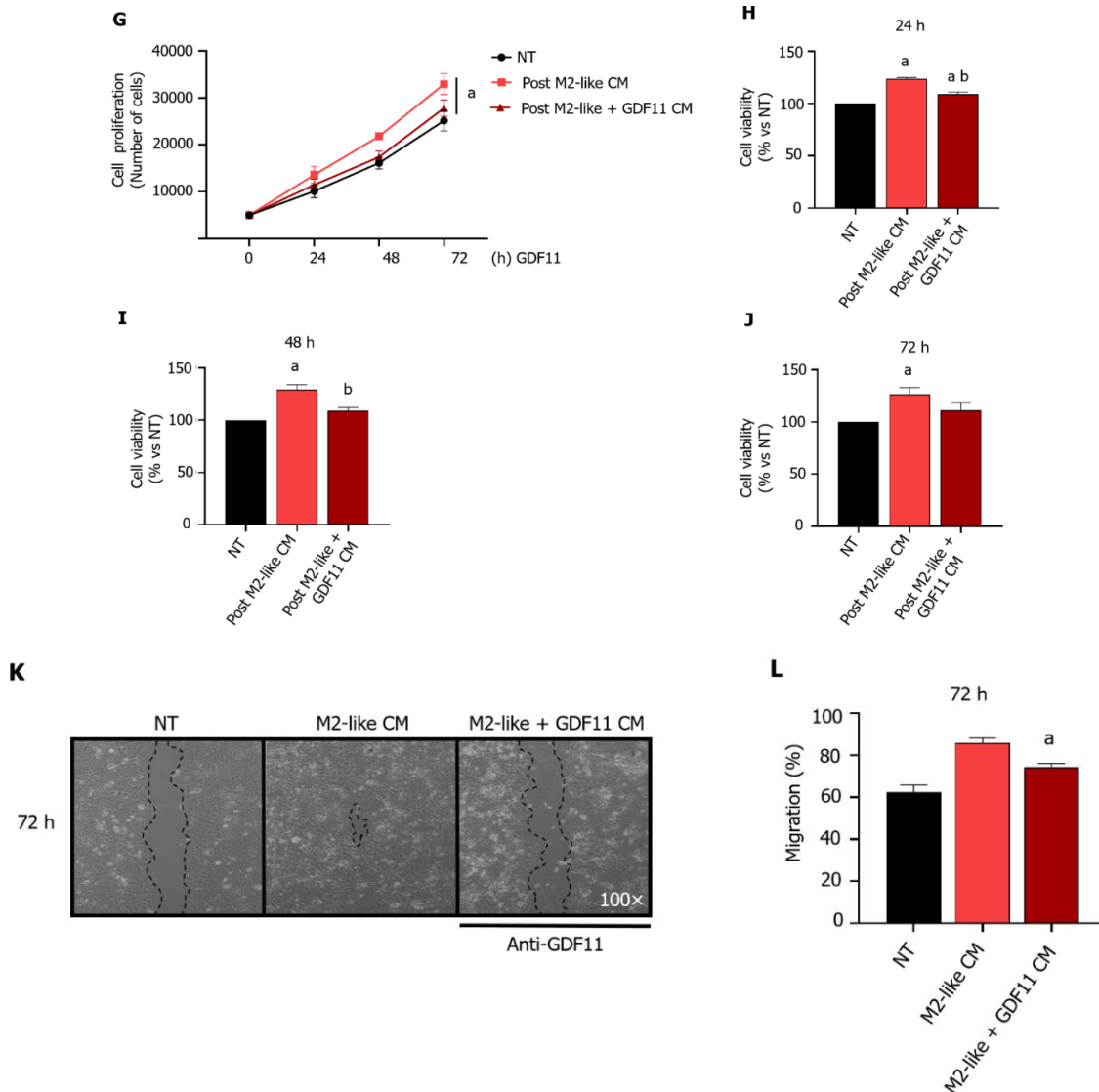
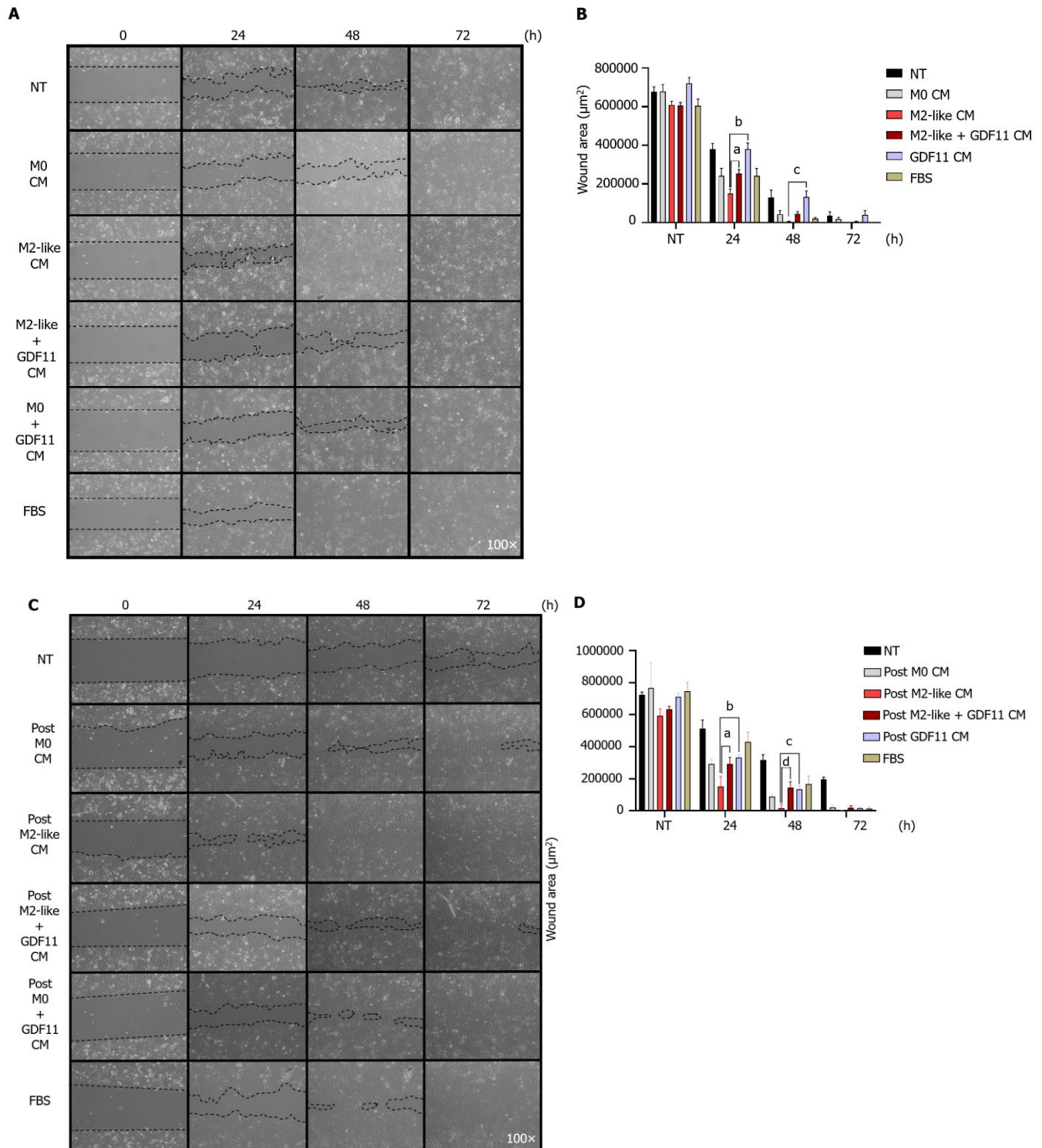


Figure 15. SHORT VERSION DATA OF CM derived from GDF11-treated macrophages delays migration capacity and proliferation in Huh7 cells. A, D, and K: cell migration capacity determined by a wound healing assay. Representative images of at least three independent experiments, 100 $\times$ of original magnification; B, C, E, F, and L: percentage (%) of migration capacity determined by Image J software. Each column or point represents the mean $\pm$ SEM of at least three independent experiments carried out in triplicate. ^a $P \leq 0.05$ vs M2-like CM or Post M2-like CM. G: Proliferation by number of cells using CCK8; H, I, and J: Cell viability by CCK8 at 24 h, 48 h, and 72 h under different treatments. Each column or point represents the mean $\pm$ SEM of at least three independent experiments carried out in triplicate. ^a $P \leq 0.05$ vs NT, ^b $P \leq 0.05$ vs Post M2-like CM. Anti-GDF11 was used to inhibit the remnants of the rGDF11. The extended data includes additional controls for the study.

EXTENDED VERSION DATA (including pos. and neg. controls)



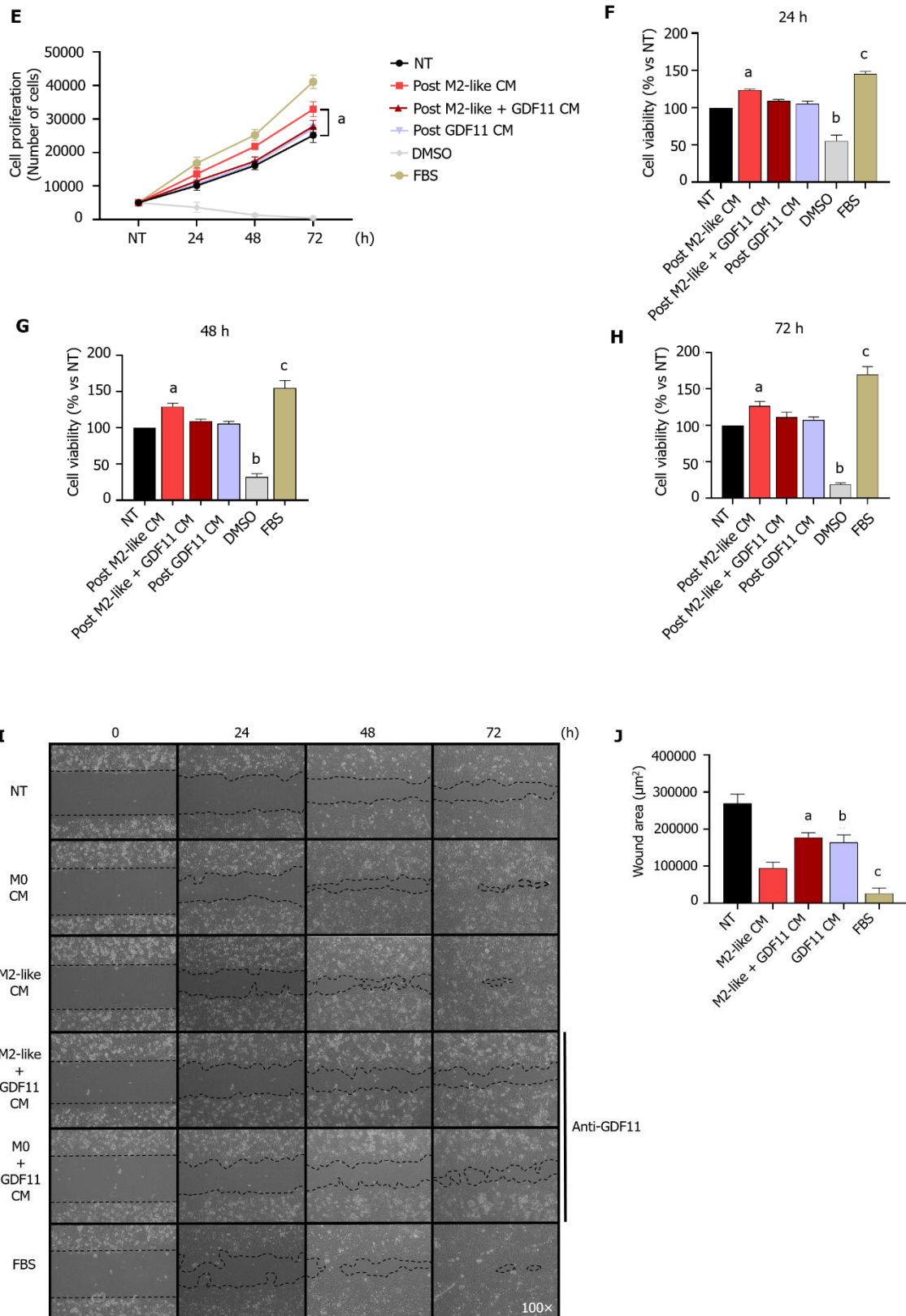


Figure 16. EXTENDED VERSION DATA OF CM derived from GDF11-treated macrophages delays migration capacity and proliferation in Huh7 cells. A, C, and I: Cell migration capacity determined by a wound healing assay. Representative images of at least three independent experiments, 100×, original magnification; B, D, and J: Wound area (μm^2) quantification determined by Image J software; each column represents the mean $\pm$ SEM of at least three independent experiments in triplicate. ^a $P \leq 0.05$ vs M2-like CM or Post M2-like CM, ^b $P \leq 0.05$ vs M2-like CM or Post M2-like CM, ^c $P \leq 0.01$ vs M2-like CM or Post M2-like CM, and ^d $P \leq 0.01$ vs Post M2-like CM. Additionally, for panel J, we compared ^c $P \leq 0.01$ vs NT. E: Proliferation by number of cells using CCK8; F, G, and H: Cell viability by the CCK-8 at 24 h, 48 h, 72 h under different treatments. Each column or point represents the mean $\pm$ SEM of at least three independent experiments carried out in triplicate. ^a $P \leq 0.05$ vs NT, ^b $P \leq 0.05$ vs NT, ^c $P \leq 0.01$ vs NT. FBS (complete media without CM) was used as a positive control, and DMSO was used as a negative control. Anti-GDF11 was used to inhibit the remnants of the recombinant GDF11.

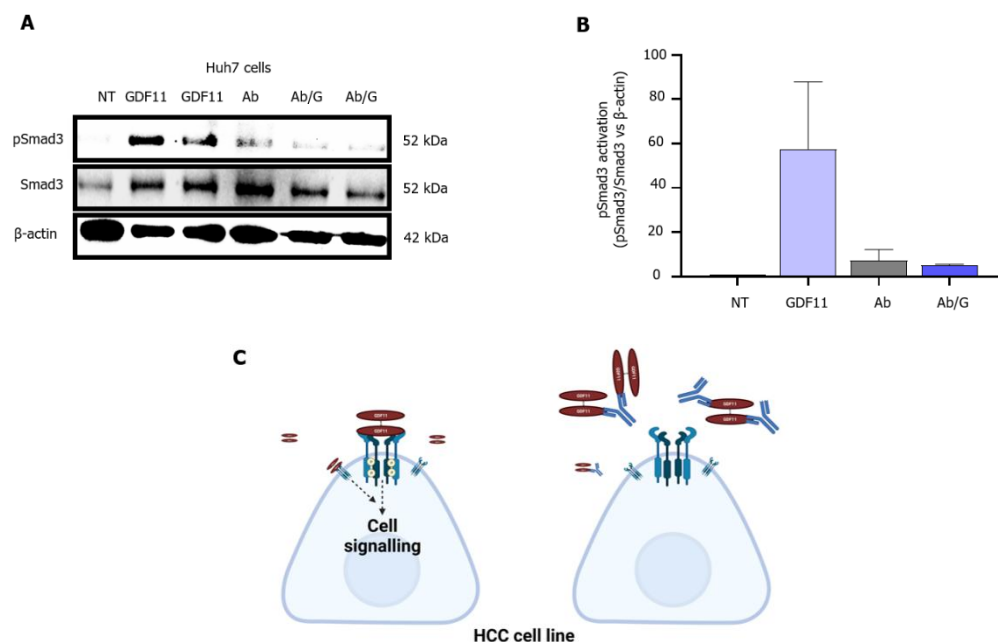


Figure 17. Anti-GDF11 turns off the phosphorylation of the Smad3 protein. A: Representative blots; and B: densitometry analysis. β -actin was used as a loading control. Ab, anti-GDF11. C: Schematic presentation of Smad pathway inhibition. Created using BioRender.com

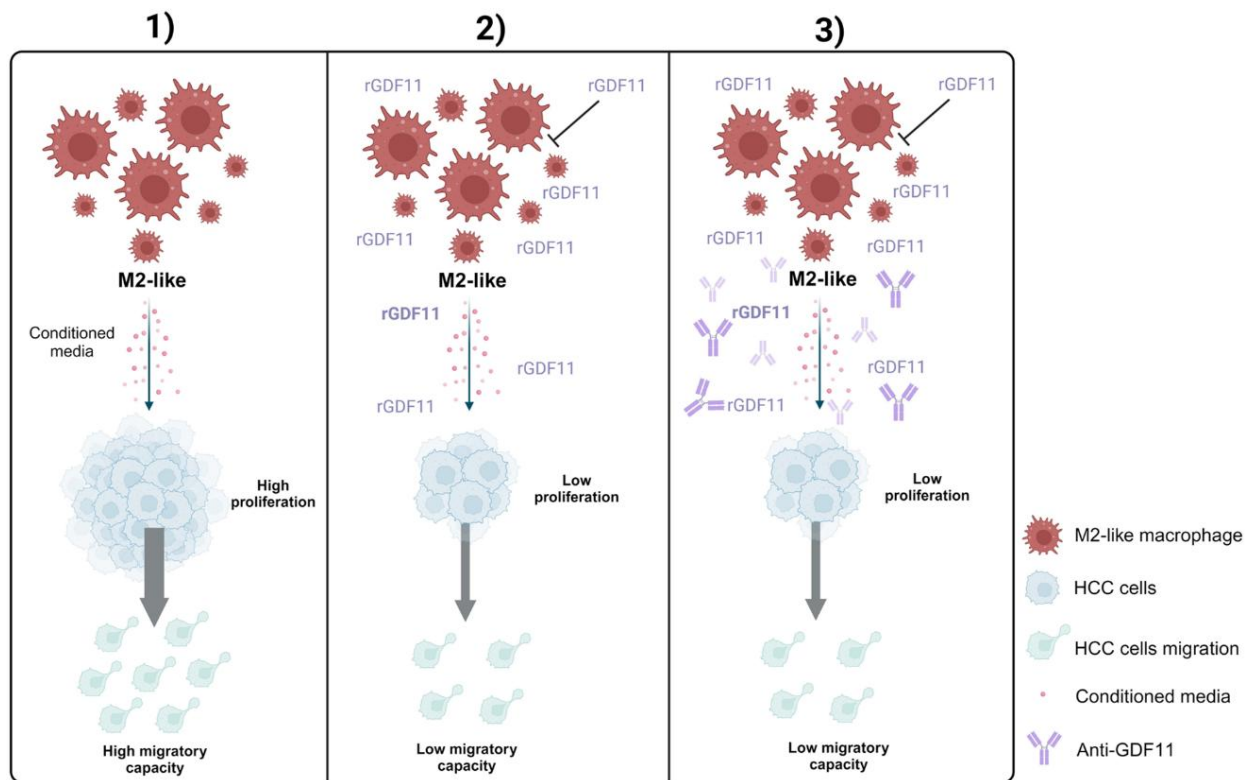
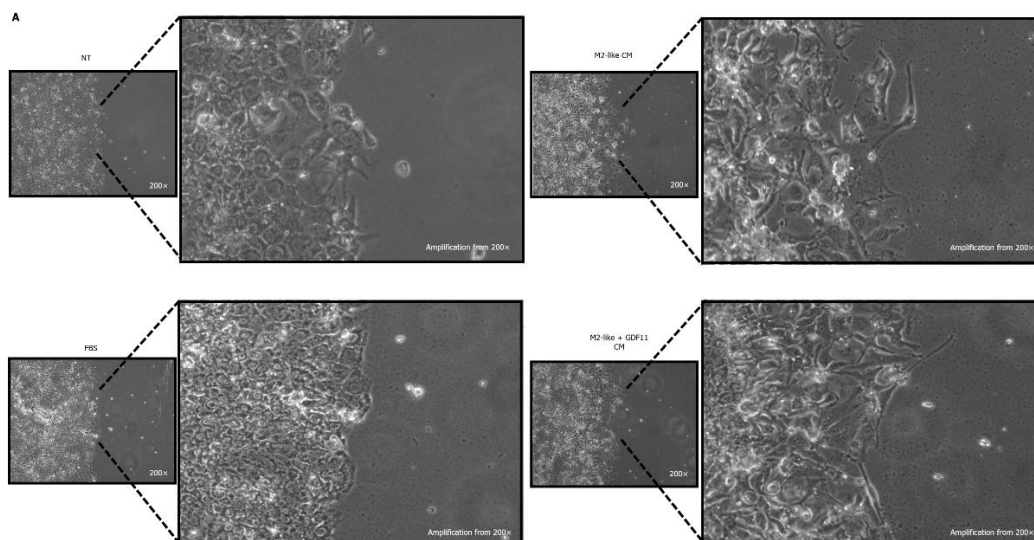


Figure 18. The three scenarios of the model. A: M2-like macrophages exacerbate aggressively on HCC cells due to secretion factors (number 1); B: GDF11 alters and mitigates M2-like polarization, reducing aggressiveness on cancer cells (number 2); and C: Using anti-GDF11 in CM did not affect the previous results on the Huh7 cell line; HCC aggressiveness is reduced (number 3). Image created using Biorender.com.



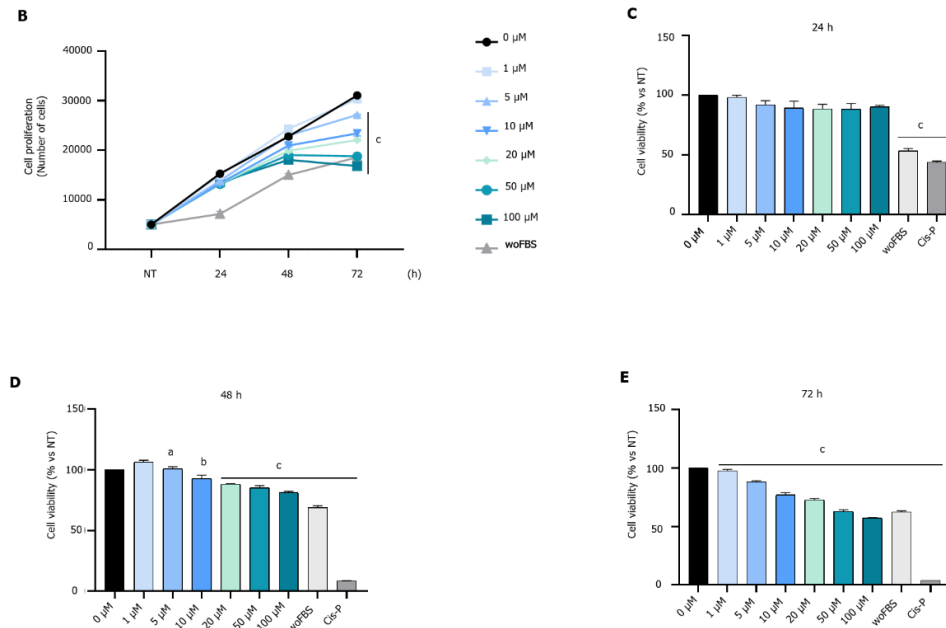


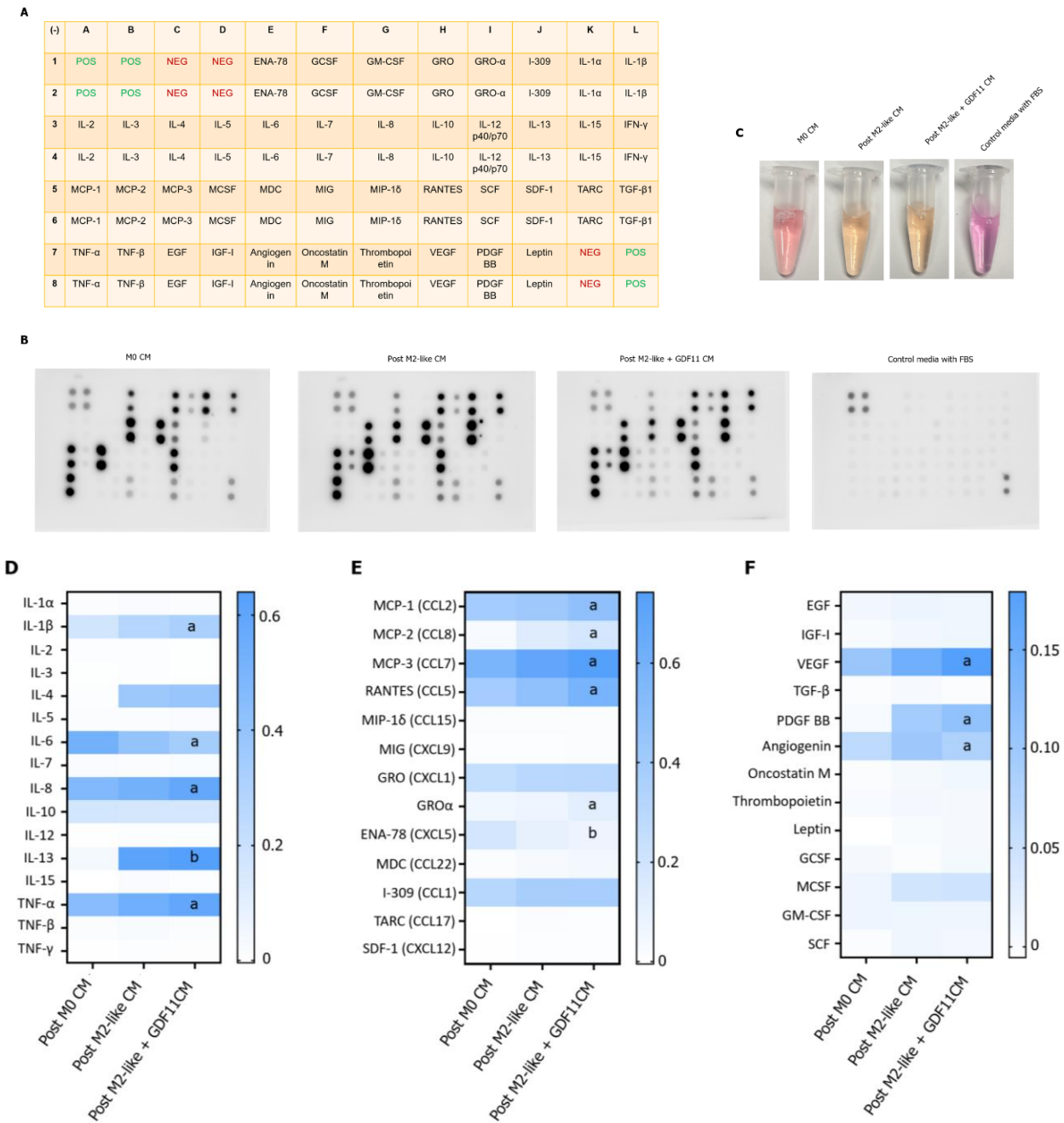
Figure 19. Microscopic evidence of the migratory process. A: Border of Huh7 wound healing cultures. Representative images of at least three independent experiments, 200 $\times$, original magnification, and amplification from the original; B: Proliferation assay by CCK8 using AraC at different concentrations; C, D, and E: Cell viability by CCK8 using AraC at different concentrations. Absence of FBS (woFBS) and cisplatin were used as negative controls, respectively. Each column represents the mean $\pm$ SEM of at least three independent experiments in triplicate. ^a $P \leq 0.05$ vs NT, ^b $P \leq 0.001$ vs NT, and ^c $P \leq 0.0001$ vs NT.

9.10 Changes in cytokine profile by GDF11 on post-M2-like macrophages.

Our last goal was to determine the cytokine profile in the CM derived from GDF11-treated and untreated macrophages. As we expected, we achieved identifying significant changes in specific secretome-treated macrophages, but not in the 42 targets.

The cytokine array showed that the content of interleukins, chemokines, and growth factors was differentially affected by GDF11 treatment in Post M2-like macrophages (**Figure 20**). Notably, we noted that GDF11 reduced the expression of IL-6, ENA-78, and angiogenin, which are key

proteins in the promotion of aggressiveness in many tumors. However, we found an increase in other molecules such as IL-1 β , IL-8, IL-13, TNF- α , MCP-1, MCP-2, MCP-3, RANTES, GRO α , VEGF, and PDGF-BB, which could execute a pro-inflammatory profile (**Figure 20D-I**).



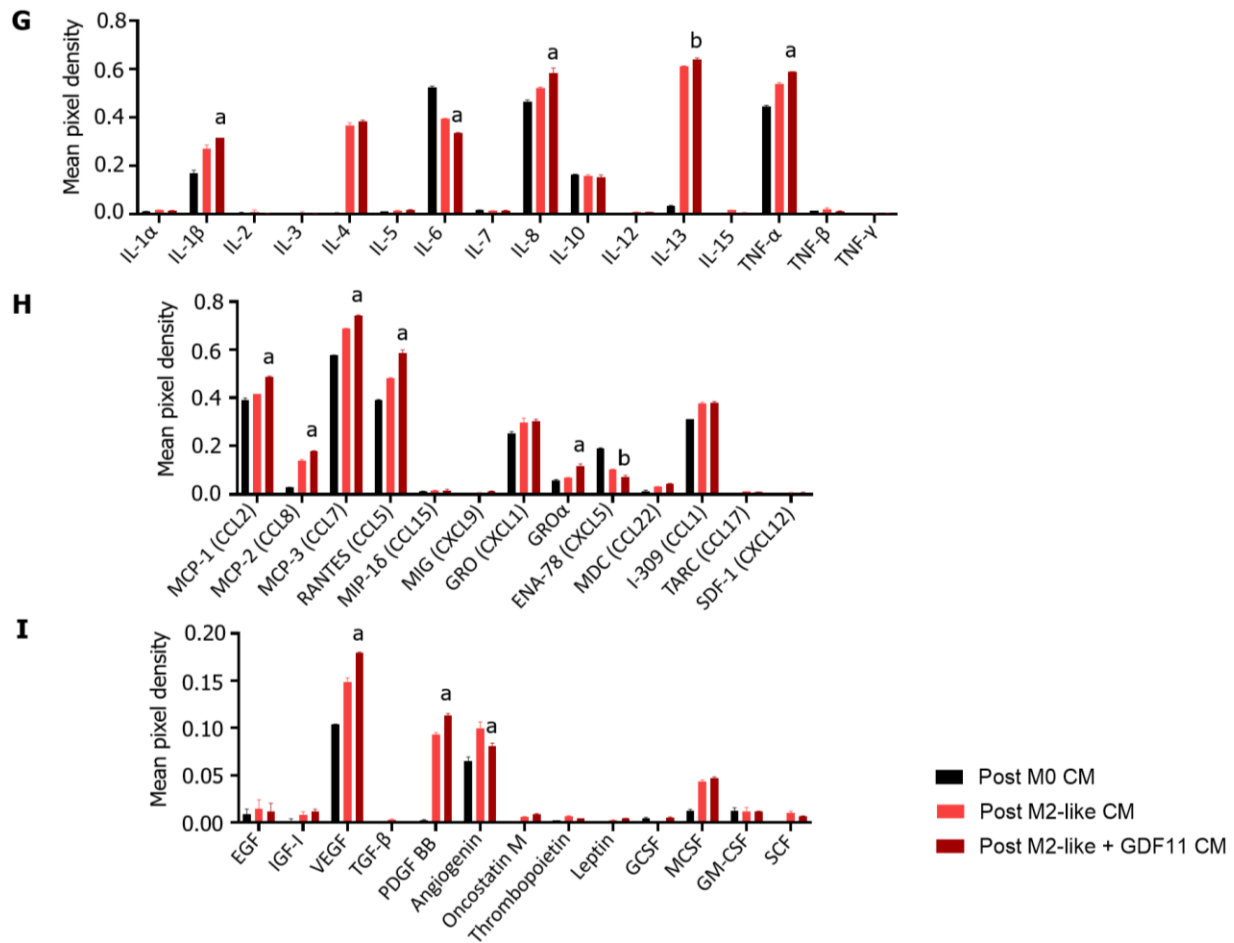


Figure 20. Cytokine profile changes induced by GDF11 on Post M2-like macrophages. A: The specific location (map) of cytokines in the cytokine array membranes; B: Representative membranes or dot blots; C: CM quality and visual acidification status derived from M0, Post M2-like, and Post M2-like + GDF11 macrophages; D and G: Interleukin and interferon family alteration by GDF11 on Post M2-like macrophages. ^a*P* < 0.0001 vs Post M2-like CM, ^b*P* = 0.0012 vs. Post M2-like CM; E, and H: Chemokine family alteration by GDF11 on Post M2-like macrophages. ^a*P* < 0.0001 vs Post M2-like CM, ^b*P* = 0.0050 vs. Post M2-like CM; F, and I: Growth factors alteration by GDF11 on Post M2-like macrophages. ^a*P* ≤ 0.0001 vs Post M2-like CM. The mean pixel density in each column or on the heat map represents the mean ± SEM of at least two independent experiments.

10. DISCUSSION.

Today, there is enough evidence that GDF11 is relevant in cancer diseases, owing to downregulation in many tumors, including breast, pancreatic, and liver cancers, and mentions the display of antitumoral properties (Bajikar et al., 2017; Y. Liu et al., 2018; Zhang et al., 2023). Also, our group has previously reported these anti-tumoral properties of GDF11 in HCC-derived cells. GDF11 mitigates aggressiveness parameters, including proliferation, migration, invasion, bioenergetics, and lipid metabolism (Gerardo-Ramírez et al., 2019; Hernandez et al., 2021). Moreover, GDF11 obstructs the malignant progression by regulating the mTORC1-autophagy axis or inducing apoptosis through the ROS-JNK pathway (Wu et al., 2024; Zhang et al., 2023). More evidence is shown in our last report, in which the aggressiveness of T-cell acute lymphoblastic leukemia-derived cells was mitigated with GDF11 due to the reduction in the migration process and the transcription factor forkhead box P3 (FOXP3), an aggressive marker (Hori, 2021), demonstrating the new impact beyond carcinomas (SÁNCHEZ-RODRÍGUEZ et al., 2025).

These discoveries prompted us to investigate whether GDF11 influences additional leukocyte populations from the TIME, including TAMs. This idea emerged following the detection of high M2-like macrophage (CD206+) infiltration in liver tumors derived from MASH etiology (Simoni-Nieves et al., 2021).

TGF- β , produced by TAMs, is also an inducer of M2-like polarization through the canonical Smad2/3 pathway, and here starts the dilemma. (Chen et al., 2022; Gungor et al., 2022; Z. Liu et al., 2018; Zhou et al., 2023). GDF11 belongs to the same family and uses the same signaling but with anti-tumoral properties (Gerardo-Ramirez et al., 2023), as we described in our study.

The great relevance and justification for the use of this monocytic cell line, such as THP-1, is that it is a suitable model for macrophage research to simulate immunological effects, such as macrophage polarization. Besides, THP-1 more closely mimics monocyte-derived macrophages than other human myeloid cell lines (Auwerx, 1991; Sharma et al., 2024). On the other hand, they are stimulated by various factors to mimic M2-like polarization, such as IL-4/13, thereby obtaining a simpler and purer model compared to using CM from other cancer cells (Bhattacharjee et al., 2013). Additionally, monocyte-derived macrophage density is higher than that of resident macrophages in the early and late stages of HCC (Degroote et al., 2021).

Additional reasons to work with TAMs in HCC are that KCs comprise 80-90% of all macrophages in the body and cover up to 20% of non-parenchymal cells in this organ (Terai et al., 2017). Other interesting evidence is that TAMs can comprise up to 50% of the tumor mass but vary between tumor stage and features (Keren et al., 2018; Wang et al., 2024). Moreover, they provide cholesterol to cancer cells (Goossens et al., 2019) to support tumor progression because it is a fundamental lipid for cell membranes, and others, such as hydroxycholesterol, act as a signal to regulate tumor aggressiveness (Marengo et al., 2016). In our research, we found a reduction in cholesterol content, suggesting this alteration in paracrine communication by GDF11.

Although the effects on ROS and CD206 marker were reduced, our data may only suggest a reduction in pro-tumoral behavior, but not clear M1-like polarization. M1-like macrophages are more proficient ROS generators than M2-like macrophages, which can engage tissue repair (Deng et al., 2019; Griess et al., 2020). The last fact may elucidate the mitigation of migration in the wound closure observed in GDF11-treated macrophage CM. Conversely, the simple loss of CD206 and CD163 markers in many cancers has been associated with a favorable prognosis

for patients, according to the high overall survival rates described in intrahepatic cholangiocarcinoma, HCC, and other tumors (Bao et al., 2019; Mao et al., 2024; Sun et al.; Yang et al., 2019).

According to our hypothesis, we also observed changes in metabolic profile with GDF11, resetting M2-like behavior to M0. The OCR finding could contradict the current literature, as mitochondria and FAO are considered the main energy sources in M2-like macrophages (Zhang et al., 2018). This may be due to cell line features, as neoplastic cells (acute monocytic leukemia) differ from bone marrow-derived macrophages (Van den Bossche et al., 2015) in their characterization compared to primary non-neoplastic cells. Additionally, numerous methods and conditions have been reported in the literature regarding the differentiation and activation of macrophages, which may impact the response due to variations in stimulation time and concentration (Nascimento et al., 2022). Therefore, our data suggest that mitochondria play an essential role in macrophage responses (Angajala et al., 2018).

In the second part of the research, we used Huh7 cells as a model for HCC cells, due to their highly aggressive features and high reports, including our research group. Besides, these Huh7 cells were used to demonstrate the effects observed in our previous publications (Gerardo-Ramírez et al., 2019; Hernandez et al., 2021), and many effects possessed similar responses when using other cell line origins. Later, we knew that the secretory response from CM of M2-like macrophages enhanced both migratory capacity and proliferation (Dong et al., 2019; Yang et al., 2018), as observed in our *in vitro* models using Huh7 cells.

Successfully, we demonstrated relevant changes in the behavior of Huh7 cells by applying the CM from GDF11-treated M2-like macrophages as the key experiments. Our result showed a neutralizing effect, rather than a reduction in the aggressiveness parameters vs. NT cells, as a

switch-off effect provided by TME. We should not minimize this because the effect on cancer has been corroborated previously, demonstrating the two-flank attacks by GDF11. In short, at this point, we wondered about the possibility of changes in the secretome in macrophages. Moreover, we cannot discard the possibility that the high ROS production by GDF11-treated macrophages hinders wound closure as a cytotoxic stimulus, as has been reported (Deng et al., 2019).

Effectively, we demonstrated that GDF11 affected the secretome of M2-like macrophages, and we focused on cytokine secretion, such as IL, chemokines, and some growth factors. The specific downregulation of IL-6, ENA-78, and angiogenin was essential to explain the loss of malignant parameters in cancer cells. IL-6 is a pleiotropic molecule with pro-inflammatory effects. As a loop, IL-6 upregulates IL-6-R, which boosts the aggressiveness of HCC (Song et al., 2023), and IL-6 also stimulates M2 polarization in other cancers (Chen et al., 2024). We showed that GDF11 reduced IL-6, indicating a mitigation of this aggressiveness. ENA-78, known as CXCL5, has been used as an indicator of tumor progression and facilitator of tumor growth and invasion (Laschtowitz et al., 2023; Ren et al., 2022), and strikingly, GDF11 has been shown to reduce this molecule in CM. Similarly, angiogenin was reduced by GDF11, which makes sense because it could be driven by IL-6, as has been reported (Hengjuan et al., 2019; Zhou et al., 2018).

The increment induced by GDF11 of additional chemokines and growth factors detected by the kit may suggest that other family molecules could also induce anti-tumoral effects in the CM derived from GDF11-treated macrophages. Unfortunately, we were unable to detect more than 42 targets. We can investigate through molecular sequencing for the subsequent research, but it is a fact that our GDF11 molecule executes secretome changes in macrophages.

These experiments could represent the future of therapy, given the limited effectiveness of current immunotherapy against specific types of liver cancer, such as MASH-HCC, the most aggressive form (Eghbali & Heumann, 2025; Pfister et al., 2021).

Furthermore, the antitumoral effects of GDF11 extend beyond transformed HCC cells, as we have described in M2-like macrophages within the TME and TIME.

Finally, more evidence has been reported regarding GDF11 in other pathologies, particularly in relation to macrophage polarization, such as severe acute pancreatitis (Duan et al., 2022) and ischemia-reperfusion-induced acute kidney injury (W.-h. Liu et al., 2023), which affected the polarization balance. Additional evidence reported that GDF11 displays as an anti-inflammatory molecule, reducing the inflammatory process in arthritis (Li et al., 2019), and controlling psoriasis-like skin inflammation (Wang et al., 2019). This research adds or contributes more evidence of GDF11, representing the first report of GDF11 in the TAMs and HCC context, and our data differ due to the experimental strategy employed.

11. CONCLUSIONS.

Our *in vitro* study provides clear and relevant evidence of GDF11 in the response of “M2-like” macrophages, sufficient to affirm GDF11's dual antitumoral effect on TAMs and HCC cells, as our research group previously demonstrated. On the other hand, we described the individual and interactive effects of both cell lines. In short, GDF11 decreases aggressiveness in HCC cells and reduces the influence of leukocyte components, such as TAMs, with M2-like polarization. This information could be important for developing new therapeutic targets to enhance current immunotherapy.

12. GRAPHICAL ABSTRACT.

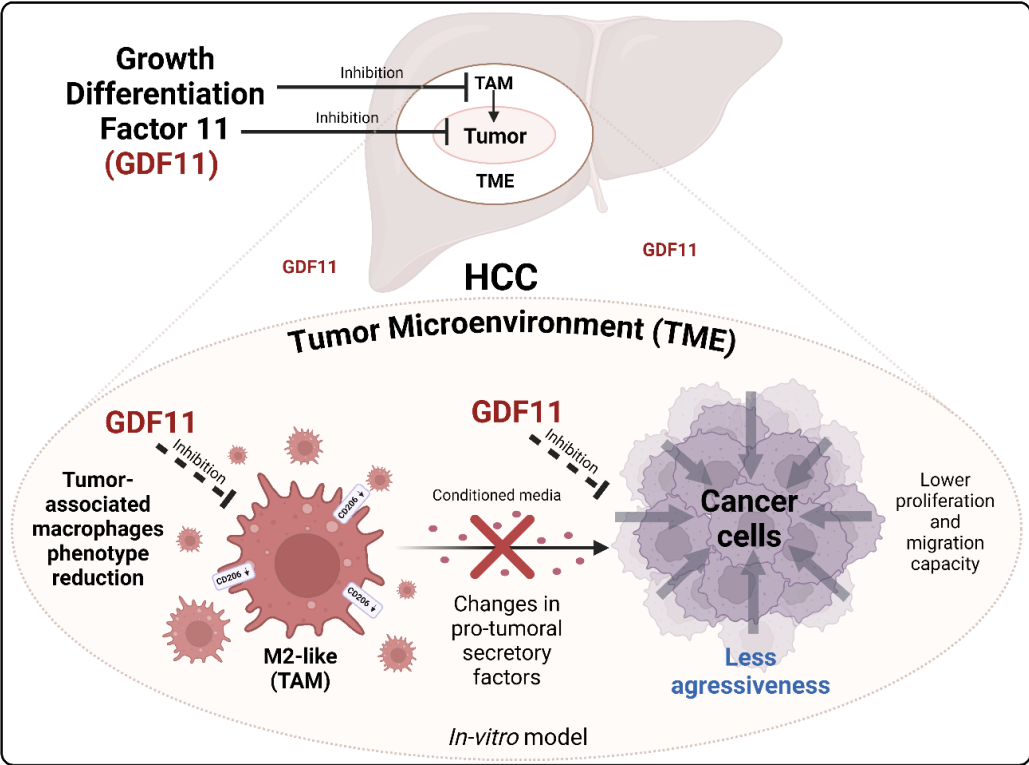


Figure 21. Graphical abstract. GDF11 mitigates M2-like macrophage polarization and reduces the pro-tumoral behavior of HCC cells. Created using Biorender.com.

13. KEY ABBREVIATUES.

ActRIIA: Activin type IIA.

ALK: Activin receptor-like kinase.

AraC: Cytosine arabinoside/ cytarabine.

BCA: Bicinchoninic acid.

BMP: Bone morphogenetic protein.

CAFs: Cancer-associated fibroblast.

CAR: Chimeric antigen receptor.

CD: cluster of differentiation.

CM: Conditioned media.

CCK8: Cell Counting Kit-8.

CSF: Colony-stimulating factor.

CTLs: Cytotoxic T lymphocytes.

DC: Dendritic cells.

DHE: Dihydroethidium.

DMSO: Dimethyl sulfoxide.

ECs: Endothelial cells.

ECAR: Extracellular acidification rate.

EDTA: Ethylenediaminetetraacetic acid

EMT: Epithelial-mesenchymal transition.

FAO: Fatty acid oxidation.

FCCP: Carbonyl cyanide-4- (trifluoromethoxy)phenylhydrazine.

FCM: Flow cytometry.

FBS: Fetal bovine serum.

GDF11: Growth differentiation factor 11.

GM-CSF: Granulocyte colony-stimulating factor.

HCC: Hepatocellular carcinoma.

HIF1 α : Hypoxia inducible factor 1.

IFN- γ : Interferon-gamma.

IL: Interleukin.

INEGI: Instituto Nacional de Estadística y Geografía.

iNK: Invariant NK.

KCs: Kupffer cells.

LCEC: Liver sinusoidal endothelial cells.

LPS: Lypopolysaccharides.

MAPK: Mitogen-Activated Protein Kinase.

MASH: Metabolic Dysfunction-Associated Steatohepatitis.

MASLD: Metabolic dysfunction-associated steatotic liver disease.

MDSC: Myeloid-derived suppressor cells.

MET: Mesenchymal-epithelial transition.

MFI: Mean fluorescence Intensity.

MHC: Major histocompatibility complex.

MoMF: Monocyte-derived macrophages.

NASH: Nonalcoholic steatohepatitis.

NOS: Nitric oxide synthase.

NK: Natural killer cells.

OCR: Oxygen consumption rate.

OPA: O-phthalaldehyde.

OXPHOS: Oxidative phosphorylation.

PBS: Phosphate-Buffered Saline.

PCSK5: Proprotein Convertase Subtilisin/Kexin Type 5.

PD1: Programmed cell death protein 1.

PD-L1: Programmed Death-Ligand 1.

PLC: Primary liver cancer.

PMA: Phorbol-12-myristate-13-acetate.

RNS: Reactive oxygen species.

ROS: Reactive oxygen species.

seqRNA: RNA sequencing.

TAMs: Tumor-associated macrophages.

Th: T helper cells

TILs: Tumor-infiltrated leucocytes.

TLS: Tertiary lymphoid structures.

TLR: Toll-like receptor.

TIME: Tumor immune microenvironment.

TME: Tumor microenvironment.

TNF: Tumor necrosis factor.

TGF- β : transforming growth factor β .

VEGF: Vascular endothelial growth factor.

WHO: World Health Organization.

14. REFERENCES.

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15. ANNEXES.
Ph.D. products

Annex I. Review paper published in the Exploration of Digestive Diseases.



Tumor immune microenvironment modulation by cholesterol in hepatocellular carcinoma

Alejandro Escobedo-Calvario^{1,2} , Lisette Chávez-Rodríguez^{1,2} , Arturo Simoni-Nieves^{2,3} , Verónica Souza-Arroyo^{2,4} , Roxana U. Miranda-Labra^{2,4} , Luis E. Gomez-Quiroz^{2,4} , María Concepción Gutiérrez-Ruiz^{2,4*} 

¹Posgrado en Biología Experimental, DCBS, Universidad Autónoma Metropolitana-Iztapalapa, Mexico City 09340, Mexico

²Área de Medicina Experimental y Traslacional, Departamento de Ciencias de la Salud, Universidad Autónoma Metropolitana-Iztapalapa, Mexico City 09340, Mexico

³Department of Biological Regulation, Weizmann Institute of Science, Rehovot 7610001, Israel

⁴Laboratorio de Medicina Experimental, Unidad de Medicina Traslacional IIB/UNAM, Instituto Nacional de Cardiología Ignacio Chávez, Mexico City 14080, Mexico

*Correspondence: María Concepción Gutiérrez-Ruiz, Área de Medicina Experimental y Traslacional, Departamento de Ciencias de la Salud, Universidad Autónoma Metropolitana-Iztapalapa, Av. San Rafael Atlixco 186, Col. Vicentina, Iztapalapa, Mexico City 09340, Mexico. mcgr@xanum.uam.mx

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Abstract

Hepatocellular carcinoma (HCC) is considered one of the most aggressive tumors worldwide. The consumption of lipid-enriched diets, mainly high cholesterol, induces oxidative stress and chronic inflammation, leading to HCC progression. Moreover, fatty acids and cholesterol could display differential responses on immune cells inside the tumor immune microenvironment (TIME). Tumor-associated macrophages (TAMs) represent one of the most critical leukocytes in the tumor microenvironment (TME) displaying pro-tumoral responses and one of the mainly cholesterol donors to cancer cells. Immunotherapy or cholesterol regulators, alone or combined, would represent an effective strategy for HCC treatment. Nonetheless, steatotic etiology from non-alcoholic fatty liver disease (NAFLD)-HCC tumors has been unexpectedly resulting in highly aggressive behavior.

Keywords

Hepatocellular carcinoma, cholesterol, inflammation, immune cells, macrophages

Introduction

Liver diseases represent one of the main challenges for health systems worldwide. Changes in human lifestyle, particularly those related to alcohol and unbalanced diet consumption, tend to increase the incidence and prevalence of liver diseases. The natural history of the liver disease starts with simple steatosis and then

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Annex II. Original paper published in the World Journal of Gastroenterology.



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ORIGINAL ARTICLE

Basic Study

Growth differentiation factor 11 modulates metabolism, mitigating the pro-tumoral behavior provided by M2-like macrophages in hepatocellular carcinoma-derived cells

Alejandro Escobedo-Calvario, Lisette Chávez-Rodríguez, Verónica Souza-Arroyo, Leticia Bucio-Ortiz, Roxana U Miranda-Labra, Felipe Masso, Araceli Pérez-Arenas, Rogelio Hernández-Pando, Jens Marquardt, María Concepción Gutiérrez-Ruiz, Luis E Gomez-Quiroz

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Alejandro Escobedo-Calvario, Lisette Chávez-Rodríguez, Verónica Souza-Arroyo, Leticia Bucio-Ortiz, Roxana U Miranda-Labra, María Concepción Gutiérrez-Ruiz, Luis E Gomez-Quiroz, Department of Ciencias de la Salud, Universidad Autónoma Metropolitana, Mexico City 09340, Ciudad de México, Mexico

Felipe Masso, Araceli Pérez-Arenas, Unidad de Medicina Traslacional, Instituto Nacional de Cardiología Ignacio Chávez, Mexico City 14080, Ciudad de México, Mexico

Rogelio Hernández-Pando, Department of Pathology, National Institute of Medical Sciences and Nutrition Salvador Zubiran, Mexico City 14080, Ciudad de México, Mexico

Jens Marquardt, Department of Medicine, University of Lübeck, Lübeck 23552, Schleswig-Holstein, Germany

Co-corresponding authors: María Concepción Gutiérrez-Ruiz and Luis E Gomez-Quiroz.

Corresponding author: Luis E Gomez-Quiroz, PhD, Department of Ciencias de la Salud, Universidad Autónoma Metropolitana Iztapalapa, No. 186 San Rafael Atlixco, Mexico City 09340, Ciudad de México, Mexico. legq@xanum.uam.mx

Abstract

BACKGROUND

Hepatocellular carcinoma (HCC) is one of the most aggressive tumors worldwide. Chronic inflammation contributes to tumor evolution, and the infiltration of tumor-associated macrophages (TAMs), also known as M2-like macrophages, is associated with the most aggressive behavior. Therefore, these macrophages provide the primary growth and migratory factors to the tumor cells, including those of HCC. Current therapies are not well optimized for eliminating transformed cells or neutralizing the tumor immune microenvironment leukocytes, such as TAMs. Growth differentiation factor 11 (GDF11) may represent a promising dual therapeutic target due to its reported anti-tumorigenic and immunomodulatory properties.



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GDF11 mitigates the pro-tumoral response of M2-like macrophages on HCC cells.

En la Ciudad de México, se presentaron a las 16:00 horas del día 19 del mes de noviembre del año 2025 en la Unidad Iztapalapa de la Universidad Autónoma Metropolitana, los suscritos miembros del jurado:

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