UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE MEDICINA

TESIS DOCTORAL

Microambiente Tumoral del Melanoma Humano

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Alba Gutiérrez Seijo

Directores

Paloma Sánchez-Mateos Rubio
Rafael Samaniego García

Madrid

UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE MEDICINA

TESIS DOCTORAL

Microambiente Tumoral del Melanoma Humano

MEMORIA PARA OPTAR AL GRADO DE DOCTORA

PRESENTADA POR

Alba Gutiérrez Seijo

DIRECTORES

Paloma Sánchez-Mateos Rubio y Rafael Samaniego García

UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE MEDICINA

TESIS DOCTORAL

Microambiente tumoral del melanoma humano

MEMORIA PARA OPTAR AL GRADO DE DOCTORA

PRESENTADA POR

Alba Gutiérrez Seijo

DIRECTORES

Paloma Sánchez-Mateos Rubio

Rafael Samaniego García

AGRADECIMIENTOS

Esta tesis, como tantas otras, ha surgido fruto del esfuerzo y de la dedicación, no solo de mi parte sino también de la de todas las personas que han estado a mi lado a lo largo del camino.

En primer lugar, me gustaría agradecer a mis directores de tesis, Rafa y Paloma, el haberme dado la oportunidad de emprender este camino. Gracias Paloma por tu sabiduría y por inculcarme tu espíritu crítico, tan útil tanto en ciencia como en la vida. Gracias Rafa por tu optimismo, que tanto hace falta en los días que los experimentos no salen, y por darme autonomía y confianza.

También me gustaría darles las gracias a todas esas personas que tras su paso por nuestro pequeño "gran" laboratorio han contribuido a esta tesis aun sin habérselo planteado. A Julia, por tu paciencia y predisposición, sin ti el laboratorio ya no es lo mismo. A Sara y Elena, mis "alarminas" que me han acompañado desde el principio y con las que los inicios de la tesis cobraron sentido. A Amaya y a Javi, gracias por vuestros conocimientos y experiencia, y por vuestra ayuda. A Mónica, Balti y Elena, por siempre estar ahí y hacerme los días más a amenos. Y en especial quiero darle las gracias a Celia, gracias por tu apoyo y tu amistad tanto en el laboratorio como fuera, sin ti no hubiera sido lo mismo.

Gracias a mi familia por su apoyo incondicional. Gracias a mis padres, que me lo han dado todo y nunca han dudado de mí. A mi madre, por su comprensión y amabilidad, siempre disponible sin reservas. A mi padre, por sus valores y su confianza ciega en mí.

Por último, y no por ello menos importante, gracias a Álvaro, mi marido, mi amigo y mi compañero de vida. Sin ti nada de esto habría sido posible.

ÍNDICE

Índice de contenido

Índice de figuras

Índice de tablas

ABREVIATURAS

A

ACT: Adoptive Cell Transfer, Transferencia Celular adoptiva

ActR: Activin Receptor, Receptor de Activina

AJCC: American Joint Committee on Cancer, Comité Conjunto Americano sobre el Cáncer

ALK: Activin Receptor-Like Kinase, Quinasa Similar al Receptor de Activina

AMH: Anti-Müllerian Hormone, Hormona Anti-Muleriana

B

BAMBI: BMP and Activin Membrane-Bound Inhibitor homolog, Homólogo de Inhibidor de BMP y Activina Unido a Membrana

BCR: B Cell Receptor, Receptor de Células B

BMP: Bone Morphogenetic Proteins, Proteínas Morfogénicas Óseas

C

CAF: Cancer Associated Fibroblasts, Fibroblastos Asociados a Cáncer

CCL: C-C Chemokine Ligand, Ligando de Quimiocina C-C

CCR: C-C Chemokine Receptor, Receptor de Quimiocina C-C

CD: Cluster of Differentiation, Clúster de Diferenciación

CFSE: Carboxyfluorescein Succinimidyl Ester, Éster de Succinimidil-Carboxifluoresceína

COX2: Cyclooxigenasa 2

CTLA-4: Cytotoxic T-Lymphocyte Antigen 4, Antígeno 4 de Linfocitos T Citotóxicos

D

DNA: Deoxyribonucleic Acid, Ácido Desoxirribonucleico

E

EAI: Efectos Adversos Inmuno-mediados

EGF: Epidermal Growth Factor, Factor de Crecimiento Epidérmico

ERK: Extracellular-Signal-Regulated Kinase, Quinasa Regulada por Señales Extracelulares

F

FFPE: Formalin-Fixed and Paraffin-Embedded, Embebidos en Parafina y Fijados con Formalina

FSH: Follicle-Stimulating Hormone, Hormona Estimuladora del Folículo

G

GDF: Growth Differentiation Factor, Factor de Diferenciación de Crecimiento

GM-CSF: Granulocyte Macrophage Colony-Stimulating Factor, Factor Estimulante de Colonias de Macrófagos y Granulocitos

I

ICP: Immune Checkpoint Inhibitors, Inhibidores de los Puntos de Control Inmune

IDO1: Indoleamina 2,3-Dioxigenasa 1

IFN: Interferón

IHC: Inmunohistoquímica

IL: Interleuquina

INH: Inhibina

J

JNK: c-Jun N-terminal Kinase, Quinasa c-Jun N-terminal

L

LDH: Lactato Deshidrogenasa

M

M-CSF: Macrophage Colony-Stimulating Factor, Factor Estimulante de Colonias de Macrófagos

MAPK: Mitogen-Activated Protein Kinases, Proteínas Quinasas Activadas por Mitógenos

MDSC: Myeloid Derived Suppressor Cells, Células Mieloides Supresoras

miRNA: micro RNA

mRNA: messenger RNA, RNA mensajero

N

NA: No Aplicable

NET: Neutrophil Extracellular Traps, Trampas Extracelulares de Neutrófilos

NF-κB: Nuclear factor kappa B, Factor Nuclear kappa B

NK: Natural Killers, Asesinas Naturales

NO: Nitric Oxide, Óxido Nítrico

P

PD-1: Programmed Death 1, Muerte Programada 1

PDL-1: Programmed Death-Ligand 1, Ligando de Muerte Programada 1

PGE2: Prostaglandina E2

PNM: Polimorfonucleares

PPAR: Peroxisome Proliferator Activated Receptor, Receptor Activado por Proliferadores Peroxisómicos

PTGS2: Prostaglandin-Endoperoxide Synthase 2, Prostaglandina-Endoperóxido Sintasa

R

RNA: Ribonucleic Acid, Ácido Ribonucleico

ROS: Reactive Oxygen Species, Especies Reactivas del Oxígeno

S

SMAD: Suppressor of Mothers Against Decapentaplegic, Supresor de Madres Contra el homólogo Decapentapléjico

Smurf: SMAD Ubiquitination Regulatory Factor, Factor de Regulación de la Ubiquitinación de SMAD

STAT: Signal Transducer and Activator of Transcription, Transductor de Señal y Activador de la Transcripción

T

TAM: Tumor Associated Macrophages, Macrófagos Asociados a Tumor

TGF: Transforming Growth Factor, Factor de Crecimiento Transformante

Th: T helper cell, Célula T Cooperadora

TME: Tumoral Microenvironment, Microambiente Tumoral

TNF: Tumor Necrosis Factor, Factor Tumoral de Necrosis

TNM: Tumor Node Metastasis, Tumor Ganglio Metástasis

Treg: T reguladoras

V

VEGF: Vascular Endothelial Growth Factor, Factor de Crecimiento Endotelial Vascular

RESUMEN

El melanoma es un tipo de cáncer que se origina en los melanocitos, células de la piel que se encargan de la producción de la melanina, un pigmento marrón que nos protege de la radiación ultravioleta (UV). El principal factor de riesgo para su desarrollo es precisamente la radiación UV del sol que induce mutaciones en el DNA de los melanocitos. Su incidencia ha aumentado en las últimas cuatro décadas, y es el cáncer más común en adultos jóvenes de 25 a 29 años. El melanoma es altamente curable si se detecta en sus etapas más tempranas (estadio I), pero el pronóstico es menos favorable en los melanomas localmente avanzados (estadio II) o cuando hay metástasis regionales (estadio III).

Los modelos actuales de progresión metastásica proponen que la invasión de células cancerígenas y las metástasis desde el tumor primario están fuertemente influenciadas por señales provenientes del microambiente tumoral (TME), en particular del contexto inflamatorio e inmune. Los macrófagos asociados a tumor (TAM) son la subpoblación de células mieloides más abundantes en los tumores. Son reguladores clave del desarrollo de los tejidos y la homeostasis, pero los tumores han adquirido la capacidad de educarlos para su propio beneficio y, por tanto, los TAM juegan un papel crucial en la supresión del reclutamiento y la función de las células T, lo que favorece el escape inmunológico del tumor.

El objetivo principal de esta tesis es analizar en detalle el contexto inmune innato y sus interacciones con otros componentes (tumor o estroma) del TME del melanoma humano. Nos hemos centrado en el análisis detallado de los TAM con el propósito de caracterizar el fenotipo que se asocia a la progresión metastásica de los pacientes con melanomas en estadios I-III y su validación como marcador pronóstico de la evolución clínica. Por otro lado, también proponemos abordar el estudio de los mecanismos que desencadenan la activación pro-tumoral y pro-metastásica en TAM. Con estos objetivos en mente, hemos puesto a punto la inmunofluorescencia multicolor, unida a la microscopía confocal, para estudiar diversos componentes inmunes y estromales, así como la expresión de quimioquinas y sus receptores en el TME del melanoma humano.

En trabajos anteriores se había identificado un perfil pro-tumoral de los TAM presentes en melanoma que se caracterizaba por la expresión de las citoquinas CCL20 / TNF / VEGFA. Para validar este grupo de proteínas como marcadores independientes con valor pronóstico, se realizó

un estudio mediante inmunofluorescencia multicolor en una cohorte de 83 melanomas primarios incluidos en parafina. Acorde con los resultados preliminares, se observó que la producción de las citoquinas CCL20/TNF/VEGFA por TAM correlacionaba con una mala supervivencia de los pacientes. Nuestros resultados validan este perfil pro-tumoral como un marcador pronóstico independiente de la evolución de melanoma.

Además, usando macrófagos derivados de monocitos, ideamos un sistema de cultivo in vitro de macrófagos con células de melanoma, que nos permitió explorar las vías reguladoras subyacentes a este fenotipo secretor de TAM, demostrando que p53 y NF-κB son responsables de co-regular los macrófagos condicionados por tumores. Estos resultados identifican una vía p53 / NF-kB única en TAM pro-tumoral que podría ser apta para reeducar a los TAM y bloquear sus funciones pro-tumorales.

Por otro lado, para identificar las vías moleculares responsables de la diferenciación de los TAM, se realizó un cribado de los biomarcadores secretados durante la interacción entre macrófago y melanoma. Se encontró que la activina A, miembro de la familia del TGFβ, juega un papel crucial en el TME, promoviendo la expresión de genes pro-tumorales y desencadenando funciones pro-invasivas e inmunosupresoras en los TAM. Además, el bloqueo de la activina en modelos xenogénicos de melanoma humano en ratones inmunodeficientes facilitó el rechazo del tumor tras la transferencia de linfocitos humanos al ratón. Por último, el cribado de dos colecciones de melanomas cutáneos independientes mostró que la activina A está enriquecida en TAM y en células de melanoma de pacientes con peores resultados de supervivencia y constituye un marcador pronóstico nuevo e independiente. Por lo tanto, en este trabajo hemos identificado la activina A como intermediario clave en las funciones pro-tumorales e inmunosupresoras de los TAM, con un potencial importante como biomarcador y como diana inmunoterapéutica.

ABSTRACT

Melanoma is a cancer that begins in the melanocytes, cells in the skin that normally make a brown pigment called melanin, which protects our skin from ultraviolet radiation. The major risk factor for most melanomas is precisely the UV rays from sunlight, which can induce mutations in the DNA of melanocytes. Melanoma incidence has grown in the last four decades, and it is the most common cancer in young adults aged 25 to 29 years. Melanoma is highly curable if detected at its early stages (stage I), but prognosis worsens in locally advanced melanomas (stage II) o when there is a regional metastasis (stage III).

Current models of metastatic progression propose that cancer cell invasion and metastasis from the primary tumor are strongly influenced by signals that come from the tumor microenvironment (TME), particularly from the inflammatory and immune context. Tumor associated macrophages (TAM) are the most abundant myeloid cell subpopulation in tumors. They are key regulators of tissue development and homeostasis; however, tumors have acquired the ability to educate them in their own advantage, and thus TAM play a crucial role in suppressing T-cell recruitment and function, and thereby favoring tumor immune escape.

The main objective of this thesis is to analyze in detail the innate immunity and its interactions with the rest of the components (tumor or stroma) of the TME of human melanoma. We have focused on the detailed analysis of TAM with the purpose of characterizing the phenotype associated with metastatic progression in patients with stage I-III melanomas and its validation as a prognostic marker of clinical evolution. On the other hand, we also propose to address the study of mechanisms that trigger pro-tumor and pro-metastatic activation in TAM. With these objectives in mind, we have coupled multicolor immunofluorescence with confocal microscopy to study various immune and stromal components, as well as the expression of chemokines and their receptors in human melanoma TME.

In our previous work we had identified a pro-oncogenic cytokine profile of TAM present in melanoma characterized with the expression of CCL20/TNF/VEGFA. In order to validate this group of proteins as independent markers with prognostic value, a study using multicolor immunofluorescence was performed in a cohort of 83 paraffin-embedded primary melanomas. In accordance with the preliminary results, it was observed that the production of these

cytokines by TAM correlated with poor patient survival. Furthermore, our results validate this pro-tumoral profile as an independent prognostic marker of the evolution of melanoma.

Additionally, using monocyte-derived macrophages, we devised an in vitro culture system of macrophages with melanoma cells, which allowed us to explore the regulatory pathways underlying this secretory phenotype of TAM, showing that p53 and NF-kB co-regulate tumor-conditioned macrophages. These results identify a unique p53/NF-kB pathway in pro-tumoral TAMs that could be targetable to re-educate TAMs to block their pro-tumoral functions.

On the other hand, in other to identify the molecular pathways responsible for TAM differentiation, a screening of the biomarkers secreted during melanoma-macrophage interactions was performed. Activin A, a member of the TGFβ family, was found to play an instrumental role in TME, promoting the expression of tumor-sustaining genes and the achievement of pro-invasive and immunosuppressive functions of TAM. Blockade of activin reduces the upregulation of part of these genes and prevents the acquisition of pro-tumoral functions, facilitating human melanoma rejection by transferred human lymphocytes in a xenograft mouse model. Remarkably, screening of two independent cutaneous primary melanoma collections showed that activin A is enriched in TAMs and melanoma cells from patients with worse outcomes and constitutes a new and independent prognostic marker. Thus, we identify activin A as a key intermediary in the pro-tumoral and immunosuppressive functions of TAM, with significant potential as a disease biomarker as well as an immunotherapeutic target.

INTRODUCCIÓN

Melanoma

El melanoma es un tipo de cáncer que se origina en los melanocitos, células de la piel que se encargan de la producción de la melanina, un pigmento marrón que nos protege de la radiación ultravioleta. Aunque el melanoma se desarrolla habitualmente en la piel también lo podemos encontrar en otras partes del cuerpo como en los ojos o en las mucosas de tejidos internos.

Incidencia

Durante las últimas tres décadas la incidencia y la mortalidad del melanoma han aumentado a un ritmo constante. La incidencia estandarizada por edad es de 11,4 por cada 100.000 habitantes en el continente europeo. En España se estima que se diagnosticarán alrededor de 7.500 casos en el año 2022 (REDECAN). Aunque el melanoma solamente supone menos de un 4% de los casos totales de cáncer de piel, es responsable del 80% de las muertes de este tipo de neoplasias (Miller and Mihm, 2006).

Factores de riesgo

El melanoma es una enfermedad multifactorial cuyo desarrollo va a depender tanto de factores externos como factores que dependen del individuo. Los factores de riesgo más frecuentes para el desarrollo de esta enfermedad son los siguientes (Rastrelli et al., 2014):

- La exposición a radiación ultravioleta. Está considerado como el factor de riesgo principal para el desarrollo de la mayoría de los melanomas. La luz solar es la principal fuente de radiación ultravioleta, aunque también destacan las cabinas de bronceado, que se ha demostrado que aumentan el riesgo de padecer melanoma (Arnold et al., 2018).

- Hipersensibilidad solar. Comúnmente conocida como alergia al sol, es una reacción inmunológica que se desencadena tras la exposición de la piel al sol. Se suele manifestar como una quemadura solar exagerada o como una erupción.

- Antecedentes familiares y personales de melanoma. Tanto las personas que ya han padecido melanoma alguna vez como aquellas con historial familiar de melanoma están en mayor riesgo de padecer la enfermedad.

- Grado de pigmentación de la piel. Aquellos individuos con la piel muy clara, que se queman o se llenan de pecas con facilidad tienen mayor riesgo de padecer melanoma.

- Múltiples nevi. Un lunar o nevus es un tumor benigno pigmentado derivado de los melanocitos. La presencia de más de 50 nevi aumenta el riesgo de desarrollar melanoma.

Pronóstico y Estadificación

El melanoma es altamente curable si se detecta en sus etapas más tempranas (estadio I), pero el pronóstico es menos favorable en los melanomas localmente avanzados (estadio II) o cuando hay metástasis regionales (III) (Balch et al., 2010). La enfermedad progresa en un 5-20% de los pacientes y se desarrollan metástasis a distancia; indicando que las células de melanoma ya se habían diseminado antes de la extirpación del tumor primario (Damsky et al., 2014).

La estadificación del melanoma se basa en un sistema desarrollado por el American Joint Committee on Cancer (AJCC) que utiliza la clasificación TNM: T (tamaño del tumor), N (afección de los ganglios linfáticos) y M (metástasis) (Tabla 1). En la actualidad, los factores pronósticos más relevantes en los estadios I-III son los siguientes: el espesor y/o nivel de invasión del melanoma (Breslow), el índice mitótico (definido como mitosis por milímetro), la presencia o no de ulceración en el sitio primario y el número de ganglios linfáticos regionales afectados, con distinción de macrometástasis y micrometástasis (Balch et al., 2009).

Sin embargo, en los estadios tempranos del melanoma la estadificación tradicional no evalúa con precisión el riesgo de recurrencia de un paciente individual y no define completamente el pronóstico de los pacientes en estadio II-III. El análisis de linfocitos infiltrantes de tumores (TIL), por métodos patológicos convencionales, es predictivo, pero no es lo suficientemente preciso para la aplicación clínica (Galon et al., 2016). Por lo tanto, un alto grado de heterogeneidad entre los pacientes dentro del mismo estadio clínico impide la identificación de los pacientes de alto

riesgo, que obtendrían el mayor beneficio de la inmunoterapia adyuvante. El pronóstico preciso es particularmente importante en pacientes con melanoma en estadios II-III para guiar la inmunoterapia adyuvante y se necesitan con urgencia nuevos biomarcadores.

Clasificación T	**Grosor (mm)**	**Ulceración / Mitosis**
Tis	NA	NA
T_1	≤ 1.00	a: Sin ulceración y mitosis < 1/mm2 b: Con ulceración o mitosis ≥ 1/mm2
T_2	1.01 - 2.00	a: Sin ulceración b: Con ulceración
T_3	2.01 - 4.00	a: Sin ulceración b: Con ulceración
T_4	> 4.00	a: Sin ulceración b: Con ulceración
Clasificación N	**N.º de ganglios metastásicos**	**Grado de afectación ganglionar**
No	0	NA
N_1	1	a: Micrometástasis* b: Macrometástasis**
N_2	2 - 3	a: Micrometástasis* b: Macrometástasis** c: Metástasis en tránsito/satélites sin metástasis nodal
N_3	4	
Clasificación M	**Localización**	**LDH sérica**
Mo	Sin metástasis distantes	NA
M1a	Piel distante, subcutánea o nodal	Normal
M1b	Pulmonar	Normal
M1c	Otras metástasis viscerales Cualquier metástasis distante	Elevada

Abreviaturas: Na, no aplicable; LDH, lactato deshidrogenasa
*Micrometástasis: se diagnostican tras biopsia del ganglio centinela.
**Macrometástasis: metástasis linfáticas clínicamente detectables y confirmadas mediante estudio patológico.

Tabla 1. Clasificación TNM del melanoma cutáneo. Adaptado de Balch et al., 2009.

Progresión del melanoma

Como se ha mencionado anteriormente, el melanoma se desarrolla a partir de un precursor melanocítico. Cuando la piel es expuesta a radiación ultravioleta, los melanocitos sufren

mutaciones en el DNA y proliferan hasta formar una neoplasia, que puede ser benigna (nevus o lunar) o maligna (melanoma). La mayoría de las lesiones malignas surgen *de novo*, es decir, tan solo un 20-30 % de los melanomas se originan a partir de nevi preexistentes.

Tras acumular sucesivas alteraciones genéticas, los melanocitos del melanoma comienzan a proliferar de forma aberrante y a expandirse de forma radial a través de la epidermis. Posteriormente se iniciaría un desarrollo vertical que comenzaría a invadir la dermis a través de la lámina basal del epitelio. A partir de aquí las células invasivas adquieren la habilidad de degradar la matriz extracelular, alcanzando los vasos linfáticos y como consecuencia los ganglios linfáticos. Las células de melanoma también pueden invadir vasos sanguíneos, sobrevivir en el torrente sanguíneo y metastatizar a órganos distantes, fundamentalmente pulmones, hígado, encéfalo y médula ósea.

A parte de los cambios genéticos o epigenéticos que ocurren en las células tumorales durante el desarrollo del melanoma, existen otros factores que van a dictaminar la progresión de la enfermedad como son las interacciones con los diferentes componentes del estroma: el microambiente tumoral (TME, del inglés “Tumoral Microenvironment”).

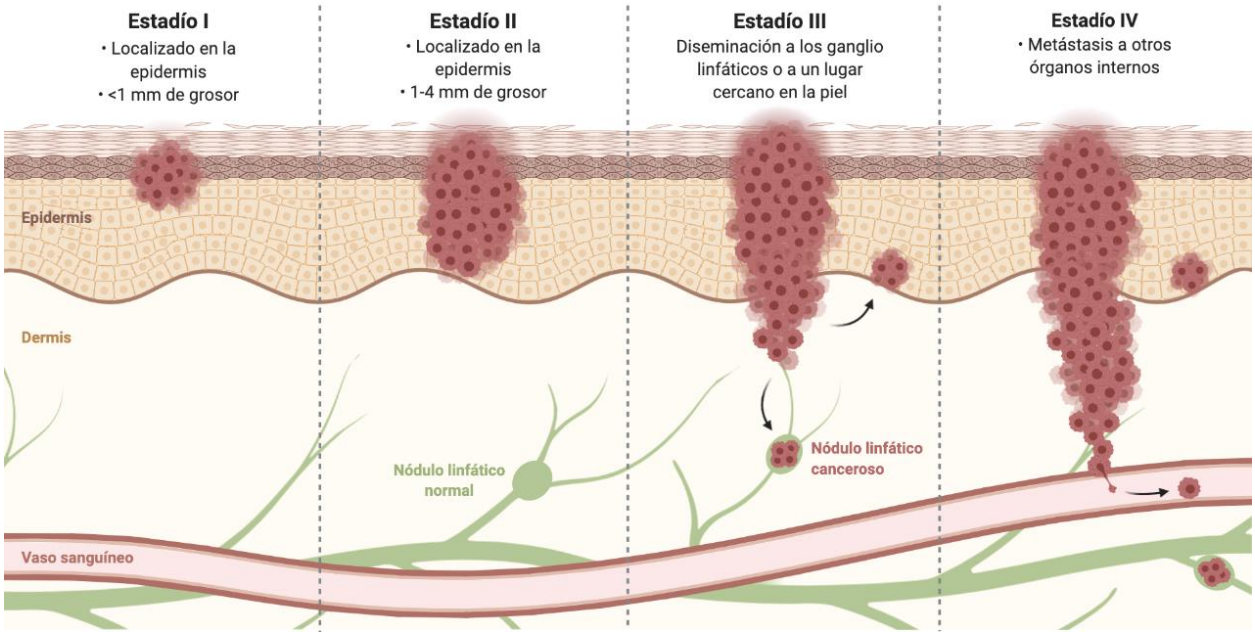


Figura 1. Representación de la progresión del melanoma según el estadio. Simplificación del sistema de estadificación TNM con cuatro estadios, del I (1) al IV (4).

Microambiente tumoral

Los tumores son organizaciones complejas de células, parecidas a los tejidos, formadas no solo por células cancerígenas sino también por el estroma, que está constituido por fibroblastos, células endoteliales, células del sistema inmune, moléculas solubles y la matriz extracelular. Los fibroblastos y las células endoteliales se encargan de mantener la integridad de los tejidos. La función de los fibroblastos es la de producir y reorganizar la matriz extracelular con proteínas como colágeno, proteoglicanos o fibronectina, entre otras. Además, también son capaces de reclutar células del sistema inmune mediante la liberación de quimioquinas. Sin embargo, cuando pasan a formar parte el tumor (fibroblastos asociados a cáncer, CAF) se activan y comienzan a secretar de forma sostenida factores de crecimiento y citoquinas que fomentan la proliferación y la propagación del cáncer (Alkasalias et al., 2018; Kalluri, 2016; Tripathi et al., 2012). Por otro lado, las células endoteliales van a aportar el soporte nutricional necesario para su crecimiento.

El melanoma es el tumor humano que más mutaciones somáticas acumula (Alexandrov et al., 2013). Estas mutaciones específicas de los tumores los hacen altamente inmunogénicos porque no están presentes en los tejidos normales y, por lo tanto, evitan la tolerancia tímica central (Ott et al., 2017). Es por ello por lo que las células inflamatorias van a tener un papel crucial en la evolución del tumor y posterior metástasis.

En un tejido normal la función del sistema inmune consiste en regular la homeóstasis del tejido, controlando la aparición de células anormales o mutadas en el mismo, y por otro lado defendiéndolo contra agentes extraños. En principio, el sistema inmune ejercería una respuesta anti-tumoral que reconocería el tumor, detendría su progreso y eliminaría las células cancerosas. Sin embargo, cuando un tumor logra desarrollarse es debido a la capacidad que adquieren las células cancerosas para eludir la respuesta anti-tumoral e incluso modificarla hasta convertirla en una respuesta pro-tumoral (Dunn et al., 2002). Esto es debido a la plasticidad que muestran las células del sistema inmune que, en respuesta a diferentes estímulos tales como citoquinas o factores de crecimiento, son capaces de tener efectos diversos e incluso contradictorios en el desarrollo tumoral.

En un tumor se pueden encontrar componentes celulares del sistema inmune innato, como las células asesinas naturales o natural killers (NK) o diversos tipos de células mieloides (neutrófilos, basófilos, eosinófilos, monocitos/macrófagos y células dendríticas) y células del sistema inmune adaptativo (linfocitos T y linfocitos B).

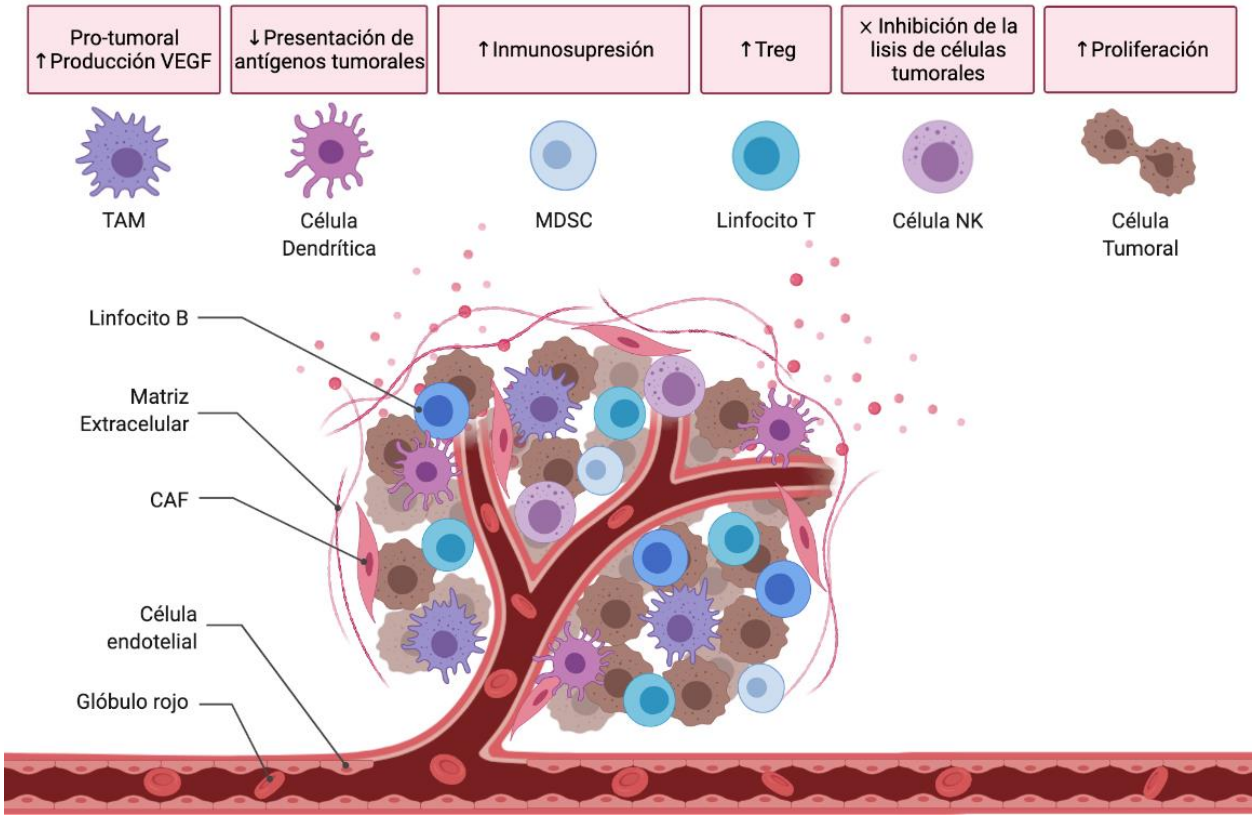


Figura 2. Microambiente tumoral. El microambiente tumoral está compuesto por diversos componentes que participan activamente en los procesos tumorales e influyen en su crecimiento y propagación. Las células inflamatorias constituyen una proporción significative del estroma del melanoma humano y proporcionan un ambiente pro-tumoral mediante la secreción de citoquinas y factores solubles.

Linfocitos T

El sistema inmune adaptativo se caracteriza por ser específico, generar memoria y proporcionar una respuesta mejorada a antígenos específicos tras un encuentro inicial. La respuesta adaptativa permite una protección a largo plazo contra antígenos específicos que puede durar semanas, meses e incluso toda la vida. Las células responsables de esta inmunidad son los linfocitos T, que provienen del timo, y los linfocitos B, que provienen de la médula ósea o “bone marrow” en inglés.

Los linfocitos T se dividen en varios subgrupos según sus funciones:

- Citotóxicos o CD8+: están especializadas en reconocer y eliminar células específicas gracias al reconocimiento de antígenos. Poseen tres mecanismos para la eliminación de células: a través de citoquinas como TNF-α e IFN-γ, mediante la liberación de gránulos citotóxicos como la granzima y la perforina y por último la inducción de apoptosis vía interacción del sistema Fas/FasL. Como cabe imaginar, su infiltración en el tumor se relaciona con un pronóstico favorable debido a su capacidad de eliminar células tumorales (Fridman et al., 2012).

- Cooperadores o CD4+: se encargan de la secreción de citoquinas que estimulan y regulan la respuesta inmune. Hay varios tipos de linfocitos T cooperadores y sus respuestas pueden ser pro-tumorales o anti-tumorales. Las células Th1 (T helper 1) se caracterizan por ayudar a los linfocitos CD8+ mediante la secreción de IL-2 e IFN-γ, colaborando en la eliminación del tumor. En cambio, las células Th2 y Th17 producen una serie de interleuquinas que generalmente promueven el crecimiento tumoral. (Fridman *et al.*, 2012).

- Reguladores: se caracterizan por la expresión de FOXP3 y CD25 (Hsieh et al., 2012). Tienen una función inmunosupresora que se ejerce a través de la producción de moléculas como IL-10, TGF-β y la expresión de CTLA-4, que en condiciones normales permite mantener la homeostasis. Sin embargo, su presencia en el tumor se asocia con peor prognosis debido a que inhiben el reconocimiento y eliminación de las células tumorales por parte del sistema inmune (Nishikawa and Sakaguchi, 2010).

Linfocitos B

Los linfocitos B expresan un receptor llamado BCR (B Cell Receptor) que cuando se activa por la unión de un antígeno específico se secreta en forma de anticuerpo y provoca como resultado final la secreción de anticuerpos específicos para ese antígeno. Estos anticuerpos se unen al antígeno en cuestión favoreciendo la eliminación del patógeno por parte los macrófagos o de las células NK. Como cabría esperar, los linfocitos B pueden ejercer un efecto anti-tumoral(Coronella et al., 2001; Milne et al., 2009), sin embargo, se ha observado que también pueden tener

respuestas pro-tumorales, secretando citoquinas que regulan a las células T citotóxicas (Yuen et al., 2016).

Natural Killers

Las NK son células linfoides innatas altamente citotóxicas que forman parte de la primera línea de defensa frente a patógenos. Aunque para muchos tipos de cáncer la presencia de NK se asocia con un buen pronóstico, su eficacia contra tumores sólidos se considera escasa debido, probablemente, al ambiente inmunosupresor característico de los tumores avanzados (Lowry and Zehring, 2017; Russick et al., 2020).

Células dendríticas

Las células dendríticas también forman parte de la respuesta innata, pero van a tener un papel clave en la iniciación de la respuesta inmune adaptativa. Se caracterizan por su capacidad de procesamiento y posterior presentación de antígenos a los linfocitos T, promoviendo su proliferación y polarización. En un ambiente tumoral, la función de las células dendríticas se suele ver disminuida o suprimida, e incluso se ha observado que son capaces de favorecer la progresión tumoral mediante la anulación de la respuesta de los linfocitos T (Fu and Jiang, 2018). Muchas de las estrategias contra el cáncer que se han desarrollado en los últimos años tienen como diana a las células dendríticas (O'Keeffe et al., 2015).

Neutrófilos

Los neutrófilos son las primeras células en acudir al sitio de infección para amplificar la señal de inflamación mediante la liberación de citoquinas. Se caracterizan por tres funciones principales: fagocitosis, desgranulación y la formación de trampas extracelulares (NET). En cáncer el papel de los neutrófilos no está libre de controversia ya que se ha asociado tanto con comportamientos pro-tumorales como anti-tumorales (Coffelt et al., 2016; Uribe-Querol and Rosales, 2015).

Células mieloides supresoras

En pacientes con tumores avanzados se pueden detectar en sangre periférica la presencia de poblaciones inmaduras de precursores mieloides que son muy inmunosupresoras y se denominan MDSC (Myeloid Derived Suppressor Cells). Su caracterización es controvertida ya que

su fenotipo es distinto en los humanos y los ratones, pero podemos diferenciar dos subpoblaciones: polimorfonucleares (PMN-MDSC) y monocíticas (M-MDSC) (Bronte et al., 2016). En la actualidad las MDSC son objeto de estudio en todos los tumores y se consideran dianas terapéuticas, ya que su presencia se corresponde con pacientes con mala evolución y peor pronóstico (Gabrilovich, 2017; Tcyganov et al., 2018).

Macrófagos

Los macrófagos son células del sistema inmune innato con un papel fundamental en la protección contra enfermedades y en el mantenimiento de la homeostasis de los tejidos. Algunas de sus funciones van a ser la fagocitosis de agentes patógenos, la presentación de antígenos y la secreción de moléculas señalizadoras (Biswas et al., 2012). Los macrófagos son el componente leucocitario más abundante del microambiente tumoral y se conocen con el nombre de macrófagos asociados a tumor (TAM, del inglés "Tumor Associated Macrophages"). Se caracterizan por una gran plasticidad que les permite adaptarse al microambiente, ejerciendo una actividad pro- o anti-tumoral dependiendo de las interacciones paracrinas que se den con las células tumorales y otros leucocitos (Locati et al., 2020), sin embargo, suelen promover la supresión inmunológica y la progresión neoplásica (Ribas and Wolchok, 2018).

Macrófagos asociados a tumor

Los TAM tienen un papel crucial en el mantenimiento e inducción del TME. Su origen puede derivar de la proliferación de los macrófagos residentes de tejido, sin embargo, es más común el reclutamiento de monocitos o MDSC a los tumores mediante la acción de determinadas quimioquinas y su posterior diferenciación a TAM (Franklin and Li, 2016; Guerriero, 2018; Lahmar et al., 2016; Qian and Pollard, 2010).

Los macrófagos se caracterizan por ser un grupo de células muy heterogéneo con muy alta plasticidad, ya que dependiendo del microambiente en el que se encuentren pueden presentar distintos estados de polarización. La polarización de los macrófagos se establece en función de la expresión de receptores, las funciones efectivas y metabólicas y en cuanto al patrón de secreción de citoquinas y quimioquinas (Biswas and Mantovani, 2010; Mantovani et al., 2002). En un esfuerzo sistemático para facilitar su estudio se ha establecido una clasificación

simplificada que identifica dos extremos funcionales: los M1 o macrófagos activados clásicamente y los M2 o macrófagos activados alternativamente. Los M1 se inducen bajo la presencia de citoquinas propias de una respuesta Th1 como IFN-γ, TNF-α o GM-CSF. Los M2 en cambio se generan en respuesta a citoquinas de tipo Th2, como son IL-4, IL-13, IL-10y TGF-β. Por lo tanto, los M1 se van a considerar proinflamatorios, produciendo altos niveles de IL-12, IFN-γ y TNF-α, mientras que los M2 van a ser antinflamatorios y producen altos niveles de IL-10.

Los TAM suelen tener un papel pro-tumoral y se han clasificado durante muchos años como macrófagos M2 debido a su menor capacidad para presentar antígenos y a su función inmunoreguladora, que ayudan a la progresión tumoral (Mantovani *et al.*, 2002; Solinas et al., 2009). Sin embargo, lo que conocemos en la actualidad sobre el papel de los TAM en el desarrollo tumoral no se ajusta a esta realidad ya que, por ejemplo, los factores citotóxicos pro-inflamatorios producidos por los M1 (TNF-α, IL1, IL6, ROS) pueden apoyar la transformación neoplásica inicial (Mantovani et al., 2008). En realidad, estas células poseen una gran heterogeneidad fenotípica y funcional que se corresponde con la variedad de estímulos que presenta el TME (Ginhoux and Guilliams, 2016; Heusinkveld et al., 2011). La coexistencia de diversas poblaciones de TAM M1 y M2 y poblaciones mixtas se describió hace tiempo en modelos tumorales murinos (Movahedi et al., 2010) y no ha sido hasta la reciente aplicación de citometría de masas que ha permitido explorar la gran heterogeneidad fenotípica y funcional de estas células en dos cánceres humanos; de pulmón y renal (Chevrier et al., 2017; Lavin et al., 2017). En el estadio inicial de cáncer de pulmón se acumulan células T disfuncionales (especialmente Treg) y macrófagos (PPARghi) en el tumor comparado con el pulmón no afectado, mientras que en el cáncer renal se han identificado 17 subpoblaciones de macrófagos, entre las que destaca una inmunosupresora (CD38+, CD204+, CD206neg) y otra asociada a peor pronóstico de los pacientes (CD163+, CD204+, CD206+, CD169+). Otros estudios en humanos tan solo caracterizan el fenotipo “pro-tumoral” de los monocitos o de las MDSC de sangre periférica de pacientes con cáncer (Chittezhath et al., 2014).

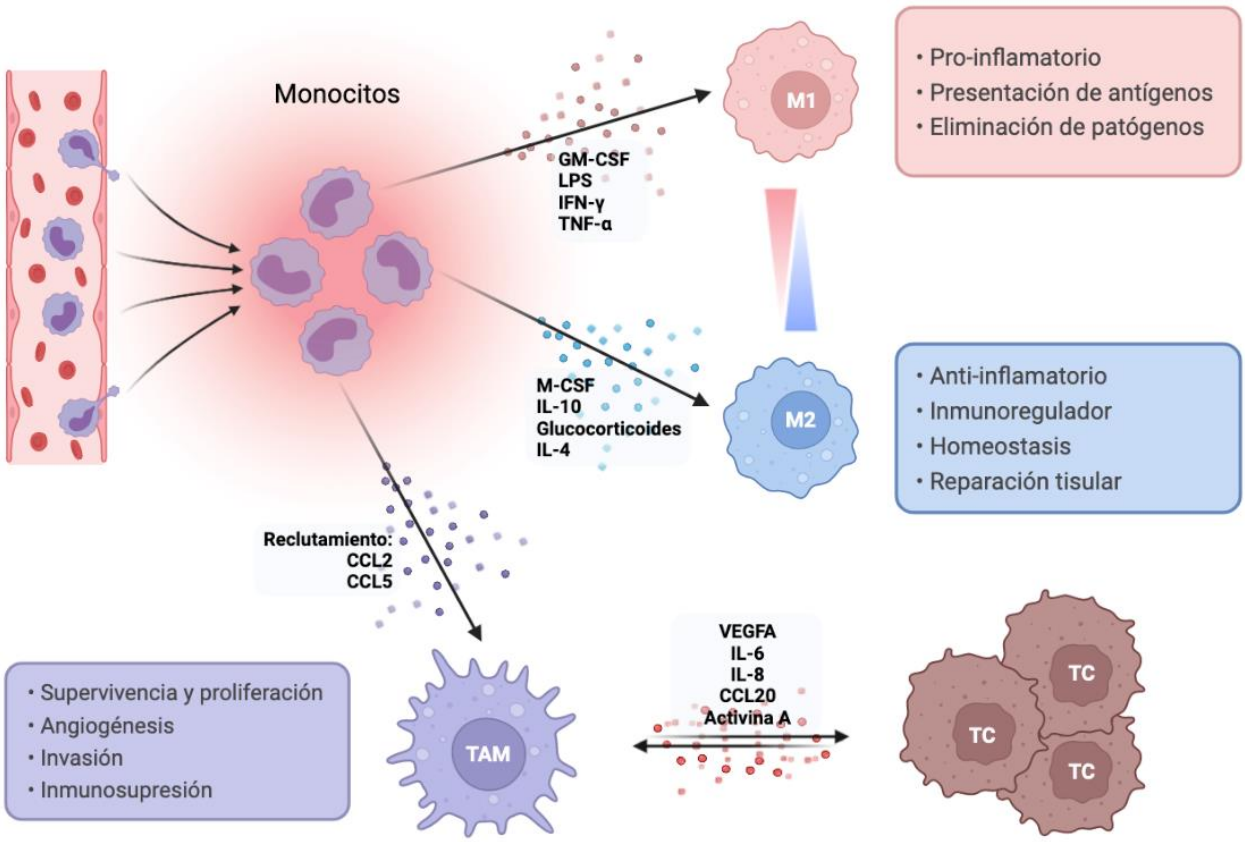


Figura 3. Estados de polarización de macrófagos. En la figura se muestran las señales y citoquinas responsables de la polarización de los macrófagos, así como las características fenotípicas y funcionales de cada tipo. En el caso de los TAM además se muestra una parte de las interacciones reguladoras existentes entre las células tumorales (TC) de melanoma y los TAM en el microambiente.

Algunas de las funciones pro tumorales más estudiadas de los TAM son las siguientes:

- **Supervivencia y proliferación de la célula tumoral**. Los TAM son capaces de producir mediadores que promueven directamente el crecimiento del tumor e incluso protegen a las células tumorales de estímulos inductores de apoptosis (Jinushi et al., 2011). Uno de estos mediadores es IL-6, que a través de la vía de STAT3 controla la proliferación y la apoptosis (Liu et al., 2010). Otra citoquina con un papel importante en la supervivencia es el TNF, que, aunque en grandes cantidades pueda inducir necrosis, se ha visto que cuando es secretado por TAM estimula la proliferación y la angiogénesis (Balkwill, 2006).

- **Invasión de la célula tumoral**. Los macrófagos tienen la capacidad de reparar y remodelar tejidos mediante la producción de metaloproteasas y otras enzimas

proteolíticas. En el microambiente tumoral esto les permite degradar la matriz extracelular, lo que contribuye a la invasión y posterior diseminación a través de los vasos sanguíneos (Mantovani et al., 2006). La secreción de factores como EGF también promueve la migración de las células tumorales (Quail and Joyce, 2013; Wyckoff et al., 2007).

- **Angiogénesis**. La vascularización es un proceso esencial en el desarrollo de los tumores sólidos y los TAM contribuyen a ella mediante la secreción de factores angiogénicos como VEGF, IL8 y CCL8. En varios modelos de tumor en los que se eliminaron los macrófagos, observaron una disminución en la angiogénesis (Gazzaniga et al., 2007; Halin et al., 2009).

- **Inmunosupresión**. Los TAM tienen la capacidad de suprimir la respuesta inmune anti-tumoral a través de la producción de citoquinas inmunosupresoras como IL10, TGF β y PGE_2, que afectan directamente a las funciones de los linfocitos CD8 y CD4 (Chen et al., 2014; Ruffell et al., 2014). Además, los TAM producen citoquinas como CCL2 para el reclutamiento de otras células inmunosupresoras como las MDSCs y las Treg (Chang et al., 2016). Por último, también se ha observado que los TAM pueden expresar ligandos de los puntos de control inmunitario ("Immune checkpoints" en inglés), como PD-L1 o proteínas de la familia B7, que se van a unir a PD-1 y a CTLA-4 respectivamente (Li et al., 2018; Prima et al., 2017). Estas rutas son mecanismos de control de la respuesta inmune y son la diana de muchos de los tratamientos de inmunoterapia actuales.

Macrófagos asociados a tumor en melanoma cutáneo

Los TAM se encuentran entre las células inmunes más frecuentes que infiltran los tumores sólidos, y muestran una variedad de fenotipos y funciones (Yang et al., 2018a). Los TAM acumulados en tumores sólidos avanzados están preferentemente sesgados hacia M2, sin embargo, los melanomas cutáneos primarios son generalmente tumores no avanzados en el momento del diagnóstico. Varios estudios han evaluado el contenido en TAM como factores pronósticos para melanomas en estadio I / II con resultados opuestos. Jensen y col. (Jensen et al., 2009) utilizando el marcador general de macrófagos humanos CD68 mostraron que la

infiltración de macrófagos en el frente invasor era un predictor independiente de mala supervivencia. Otro estudio a gran escala (202 pacientes) no corroboró la correlación entre el número de TAM, identificados con CD68, y la supervivencia del melanoma (Storr et al., 2012).

Biomarcadores

Un biomarcador es cualquier sustancia, estructura o proceso que se pueda medir en un organismo y que permita detectar enfermedades o los procesos de las mismas. Los biomarcadores pueden comprender ácidos nucleicos (DNA, RNA o miRNA), proteínas, péptidos, azúcares, lípidos o células completas. Además, diferentes partes de un organismo pueden usarse para detectar biomarcadores, como por ejemplo la sangre o sus componentes (plasma o suero), secreciones corporales, como la orina o la saliva, o tejidos de los distintos órganos.

En cáncer los biomarcadores son cada vez más estudiados y podemos encontrar tres tipos: de diagnóstico, que permiten determinar que pacientes padecen la enfermedad; de pronóstico, que ayudan a predecir el progreso de la enfermedad; y predictivos, que pueden predecir la respuesta de un paciente a un tratamiento específico.

En melanoma, a pesar de los avances en nuevas terapias, solo algunos de los pacientes que padecen esta enfermedad en estadios más avanzados se logran beneficiar de manera duradera de estas terapias, siendo solo un 16% la supervivencia a 5 años. En la actualidad el indicador de pronóstico más importante en melanoma primario es el Breslow, sin embargo, incluso pacientes con melanomas muy finos pueden llegar a desarrollar metástasis. Dada la mala prognosis para el melanoma avanzado, urge determinar nuevos biomarcadores de pronóstico para poder identificar aquellos pacientes que se puedan beneficiar de terapias más agresivas (Grivennikov et al., 2010; Pages et al., 2005).

Dentro del microambiente tumoral podemos encontrar dos fuentes de biomarcadores: los distintos tipos de células infiltradas y los factores que secretan estas células y las células tumorales. Todas las células expresan marcadores característicos que pueden ser utilizados para identificar y caracterizar los distintos tipos celulares (Biswas and Mantovani, 2010; Zhang et al., 2003). Como ya se ha mencionado anteriormente, la infiltración de las distintas células del sistema inmune puede tener consecuencias tanto en la evolución tumoral como en la

supervivencia del paciente. Además del impacto clinicopatológico que tiene la infiltración de estas células a nivel individual, es importante observar el ambiente en el que se encuentran para entender la realidad de las condiciones que se dan dentro del tumor (Hiraoka et al., 2006; Sica and Bronte, 2007).

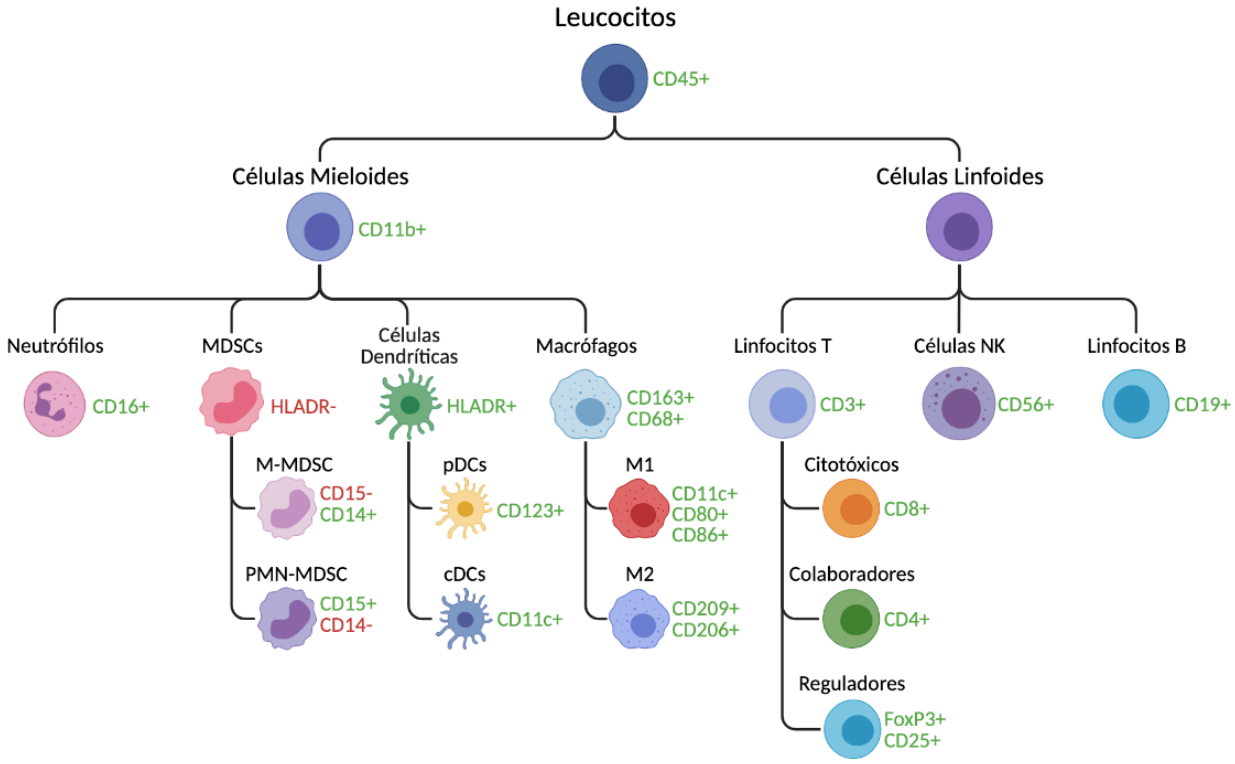


Figura 4. Marcadores del infiltrado inmune tumoral humano para inmunohistoquímica.

Activina A

La superfamilia de TGF-β (del inglés “transforming growth factor beta”) es un grupo de factores de crecimiento que juegan papeles cruciales en el desarrollo y la homeostasis de los organismos, especialmente en la embriogénesis, el crecimiento y la reparación de tejidos (Hedger and de Kretser, 2013). Estas proteínas están altamente conservadas a través de las especies y pueden señalizar tanto de manera autocrina como paracrina. En humanos se han identificado más de 30 miembros de la familia, incluyendo las proteínas morfogénicas del hueso (BMPs), los factores de diferenciación del crecimiento (GDFs), la hormona antimulleriana, la proteína nodal, las activinas y, como su nombre indica, TGF-β (Feng and Derynck, 2005). Todas estas proteínas tienen una estructura muy similar y señalizan a través de complejos

heterotetraméricos formados por una combinación de los siete receptores de tipo I y los cinco receptores de tipo II (Tabla 2) (Heldin and Moustakas, 2016).

Subfamilia	Ligandos		Receptores de tipo I	Receptores de tipo II	R-SMAD
TGF-β	TGF-β1 TGF-β3	TGF-β2	ALK1 ALK2 ALK5	TβRII	SMAD1 SMAD2 SMAD3 SMAD5 SMAD8
BMP	BMP2 BMP3 BMP4 BMP5 BMP6 BMP7	BMP8A BMP8B BMP10 BMP11 BMP15	ALK1 ALK2 ALK3 ALK4 ALK5 ALK6 ALK7	BMPR2 BMPR2B ActR-IIA ActR-IIB	SMAD1 SMAD2 SMAD3 SMAD5 SMAD8
GDF	GDF1 GDF2 GDF3 GDF5 GDF6	GDF7 GDF9 GDF10 GDF11 GDF15	ALK1 ALK3 ALK4 ALK5 ALK6	BMPR2 ActR-IIA ActR-IIB	SMAD1 SMAD2 SMAD3 SMAD5 SMAD8
Activina	Activina A Activina B Activina AB		ALK2 ALK4 ALK7	ActR-IIA ActR-IIB	SMAD1 SMAD2 SMAD3 SMAD5 SMAD8
Inhibina	Inhibina A Inhibina B		-	ActR-IIA ActR-IIB BMPR2 BMPR2B	-
Nodal	Nodal		ALK4 ALK7	ActR-IIA ActR-IIB	SMAD2 SMAD3
AMH	AMH		ALK2 ALK3 ALK6	AMHR2	SMAD1 SMAD5 SMAD8
Lefty	Lefty1 Lefty2		-	ActR-IIA ActR-IIB	-

Abreviaturas: TGF-β, transforming growth factor β; BMP, bone morphogenic protein; GDF, growth and differentiation factor; AMH, anti-müllerian hormone; ALK, activin receptor like kinase; ActR, activin receptor.

Tabla 2. Rutas de señalización de la superfamilia TGF-β en humanos. Tomado de Morianos et al., 2019.

Las activinas son glicoproteínas diméricas que pertenecen a la superfamilia de proteínas TGF-β. Están formadas por homo- o heterodímeros de las distintas subunidades β, βA, βB, βC y βE, codificadas por los genes *INHBA*, *INHBB*, *INHBC* y *INHBE*, respectivamente. Hasta la fecha se han

identificado cinco activinas, de las cuales solo tres son isoformas funcionales en humanos: activina A (βAβA), activina B (βBβB) y activina AB (βAβB) (Namwanje and Brown, 2016). La activina A, con un peso de 25 kDa, es con diferencia la activina más caracterizada. Fue aislada por primera vez en 1986 e identificada como responsable de la síntesis y secreción de la hormona foliculoestimulante (FSH) en la pituitaria (Vale et al., 1986). Está involucrada en múltiples procesos biológicos como la hematopoyesis, el desarrollo embrionario, mantenimiento y pluripotencia de células madre, reparación de tejidos y fibrosis (Morianos et al., 2019).

La señalización de la activina tiene lugar a través de la formación de un complejo de receptores formado por ALK2, ALK4 o ALK7, como receptores de tipo I, y ActRIIA o ActRIIb, como receptores de tipo II. Una vez la activina se une a los receptores de tipo II, tiene lugar el reclutamiento y activación de los receptores de tipo I mediante su fosforilación. Posteriormente los receptores de tipo I fosforilarán a los mediadores intracelulares SMAD2/3 que, tras su unión a SMAD4, se traslocarán al núcleo donde regularán la transcripción de distintos genes (Massague and Chen, 2000). Cabe añadir que la activina A puede señalizar a través de otras vías no canónicas a través de ERK, p38, JNK o Wnt (de Guise et al., 2006; Morianos *et al.*, 2019; Tamminen et al., 2015), (Figura 5).

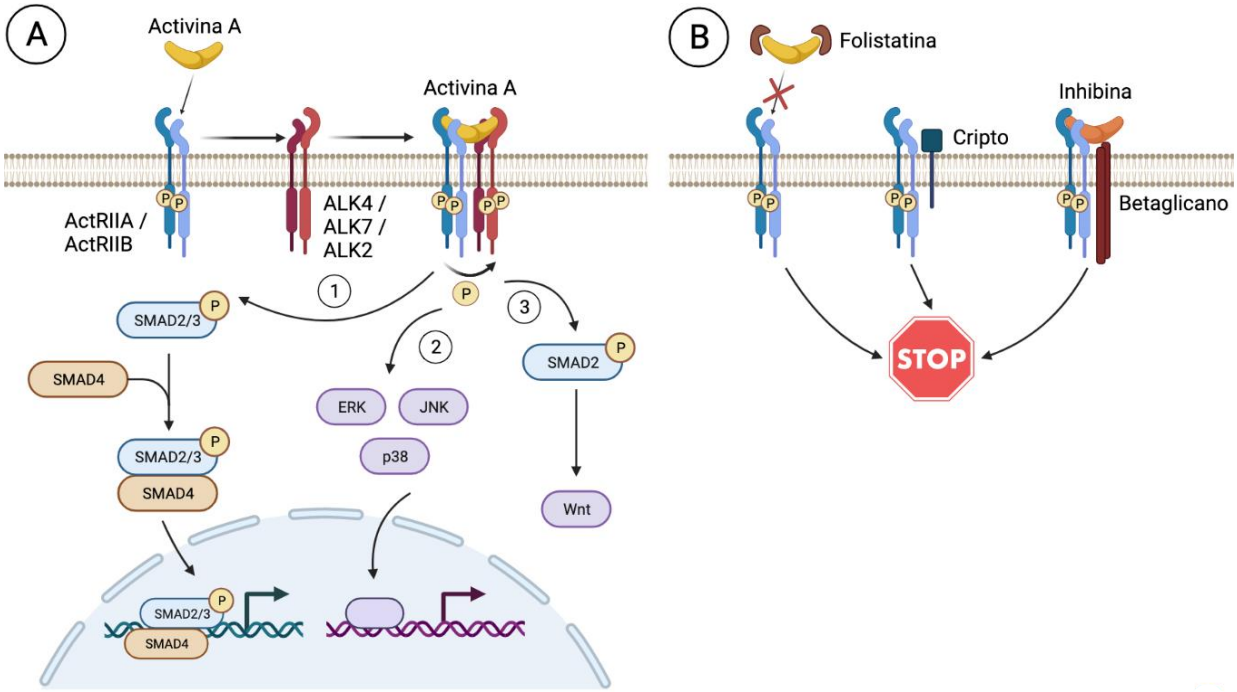


Figura 5. Señalización de la activina A. A) La activina señaliza a través de un complejo de receptores. Inicialmente se une a los receptores de tipo II, constitutivamente activados, que van a reclutar a los receptores de tipo I para su posterior fosforilación. A continuación, los receptores de tipo I fosforilan a las proteínas SMAD2/3 y éstas junto con SMAD4 transmiten la señal al núcleo. Existe además una vía de señalización no canónica a través de mediadores como ERK, p38, JNK y Wnt. B) La señalización de activina A está altamente regulada y algunos ejemplos de ello se muestran en la figura. Adaptado de Morianos et al., 2019.

Teniendo en cuenta que la activina está involucrada en múltiples procesos biológicos, su actividad está estrechamente regulada. Esta regulación puede ocurrir tanto a nivel extracelular como intracelular. A continuación, se enumeran los mecanismos de regulación de la activina más conocidos:

- La folistatina es uno de los inhibidores más conocidos y actúa uniéndose a la activina e impidiendo su interacción con los receptores.

- Las inhibinas, proteínas que forman parte también de la superfamilia de TGF-β, se unen a un receptor transmembrana llamado betaglicano, que dimeriza con receptores de activina de tipo II, inhabilitando la unión de la activina.

- BAMBI ("BMP and activin membrane-bound inhibitor homolog"). Es un pseudoreceptor que inhibe la señalización mediante la unión al receptor de tipo I.

- Cripto. Es un correceptor de nodal que actúa de forma similar a BAMBI impidiendo la interacción de activina con los receptores de tipo I.

- I-SMADs. A nivel intracelular nos encontramos con los SMAD inhibitorios, SMAD 6 y SMAD7, que se unen al receptor de tipo I una vez ha sido activado impidiendo la fosforilación de SMAD2/3. También promueven la unión de Smurf 1 y 2 ("SMAD ubiquitin regulatory factors") que inducen la degradación de los receptores por ubiquitinación.

- MicroRNAs. En los últimos años un creciente número de estudios ha demostrado que tanto la activina como sus receptores son susceptibles de ser regulados por microRNAs. Ejemplos de ellos son la inhibición del receptor ActRIIA por parte de mir-15/16 (Martello et al., 2007) o la inhibición de la expresión de *INHBA* por mir-146a (Li et al., 2016).

Activina A y sistema inmune

La activina está presente en prácticamente todos los tejidos del cuerpo humano. La observación inicial que arrojó luz a la importancia de la activina en la respuesta inmune fue la capacidad de inhibir la proliferación de los timocitos (Hedger et al., 1989). Desde entonces y tras una intensiva investigación durante las últimas décadas se ha visto que la mayoría de las células del sistema inmune, incluyendo los macrófagos, las células dendríticas, los linfocitos y las NKs, tienen la capacidad de producir y responder a la activina A, aunque de maneras muy diferentes.

En macrófagos está bien documentado que cuando un monocito o un macrófago es estimulado con activina, estas células comienzan a producir múltiples mediadores inflamatorios como IL-1β, TNF-α, IL-6, NO, PGE_2 y tromboxanos (Nusing and Barsig, 1999; Nusing et al., 1995; Yamashita et al., 1993). Otra investigación más reciente demostró que la activina, que es secretada por los macrófagos M1 o pro-inflamatorios, además suprime la secreción de la citoquina antinflamatoria IL-10 y la adquisición de marcadores característicos de los macrófagos

M2 o antinflamatorios (Sierra-Filardi et al., 2010). Sin embargo, otros estudios indican que cuando un macrófago se encuentra en estado activado, la activina A puede tener un efecto antinflamatorio como la inhibición de citoquinas proinflamatorias como TNF-α, IL-18 y IL-6 (Cuschieri et al., 2008; Sugama et al., 2007; Wang et al., 2009; Wilms et al., 2010; Zhang et al., 2005; Zhou et al., 2009). Por consiguiente, en macrófagos la activina A puede tener un efecto proinflamatorio o antinflamatorio dependiendo del estado de activación de la célula y del tipo de estímulo.

Activina A y cáncer

El papel de la activina A en el desarrollo y la progresión del cáncer es dual y va a cambiar según el contexto en el que se encuentre el tumor. Por ejemplo, en cáncer de mama se ha observado que la activina inhibe el crecimiento y promueve la apoptosis (Burdette et al., 2005; Razanajaona et al., 2007); sin embargo, también se ha visto un efecto pro-tumoral promoviendo la invasión y la metástasis (Bashir et al., 2015; Kalli et al., 2019). De la misma forma en cáncer de colon (Jung et al., 2007), de próstata (Dalkin et al., 1996; Nomura et al., 2013) y en hepatocarcinoma (Chen et al., 2000; Hyuga et al., 2000) se han obtenido resultados similares en los que la activina suprime el crecimiento, pero aumenta la migración tumoral o la metástasis.

No obstante, un aumento en la proliferación tumoral a causa de la activina se ha demostrado en cáncer de endometrio (Tanaka et al., 2004), cáncer oral de células escamosas (Chang et al., 2010) y en adenocarcinomas de esófago (Seder et al., 2009a) y de pulmón (Seder et al., 2009b).

En el caso del cáncer de piel la activina se encuentra altamente expresada en carcinomas de células basales y escamosas (Antsiferova et al., 2011; Heinz et al., 2015; Hoek et al., 2006). La sobreexpresión de activina en queratinocitos se ha observado que es tumorigénica en cáncer de piel no melanoma a través de su acción paracrina en las células del estroma, particularmente por el reclutamiento de monocitos de la sangre y su polarización hacia un fenotipo de macrófagos pro-tumorigénicos (Antsiferova et al., 2017). En modelos de ratón de melanoma con B16 se ha demostrado que la activina tiene un papel tumorigénico, reduciendo la respuesta anti-tumoral (Donovan et al., 2017; Ni et al., 2018; Rautela et al., 2019).

OBJETIVOS

1. **Identificación de poblaciones de macrófagos tumorales M1 y M2 en melanoma humano y estudio de su perfil pro-tumoral/pro-metastásico.**

    1.1. Estudio de marcadores M1-M2 in vivo.

    1.2. Correlación entre la supervivencia de pacientes y el tamaño, densidad y estado de polarización de los macrófagos tumorales.

    1.3. Determinación del perfil pro-tumoral/pro-metastásico de TAM de melanoma.

    1.4. Validación de marcadores independientes con valor pronóstico en TAM de melanoma.

    1.5. Regulación de la expresión de proteínas pro-tumorales: factores extrínsecos de señalización y vías de regulación transcripcional.

2. **Estudio de la implicación de la activina A en el microambiente tumoral del melanoma humano.**

    2.1. Analizar el papel de la activina A en el condicionamiento de los macrófagos del melanoma.

    2.2. Determinar el efecto de la activina A en la progresión del melanoma.

    2.3. Evaluar la expresión de activina A como biomarcador para el pronóstico del melanoma humano.

    2.4. Estudio del potencial de la activina A como diana terapéutica.

MATERIALES Y MÉTODOS

Los materiales y métodos empleados en esta tesis doctoral están detallados en los artículos presentados en la misma.

RESULTADOS

Los resultados de esta tesis doctoral están detallados en los artículos presentados en la misma.

Publicaciones

El fenotipo secretor de citoquinas CCL20/TNF/VEGFA de los macrófagos asociados a tumores es un factor pronóstico negativo en el melanoma cutáneo

Los TAM constituyen una gran fracción de las células inmunes infiltrantes en los tejidos del melanoma, pero su importancia para los resultados clínicos aún no está clara. Exploramos diversos parámetros TAM en muestras de melanoma cutáneo primario clínicamente relevantes, incluida la densidad, la ubicación, el tamaño y la expresión del marcador de polarización; además, debido a que la producción de citoquinas es un sello distintivo de la función de los macrófagos, medimos las citoquinas intracelulares CCL20, TNF y VEGFA mediante microscopía confocal multiparamétrica unicelular. Se utilizó el método de Kaplan-Meier para analizar la correlación con la supervivencia libre de enfermedad específica del melanoma y la supervivencia global. No se observaron correlaciones significativas con los parámetros clínicos para la densidad, morfología o ubicación de TAM. Significativamente, se cuantificaron contenidos más altos de las citoquinas intracelulares CCL20, TNF y VEGFA en los TAM que infiltran metástasis en comparación con los melanomas primarios de la piel sin metástasis ($p < 0,001$). Para explorar el mecanismo de la regulación positiva de citoquinas, realizamos estudios *in vitro* con macrófagos condicionados por melanoma, utilizando RNA-seq para explorar las vías involucradas e inhibidores específicos. Mostramos que p53 y NF-κB corregulan CCL20, TNF y VEGFA en macrófagos condicionados por melanoma. Estos resultados delinean un perfil de citoquinas pro-oncogénicas clínicamente relevante de TAM con importancia pronóstica en melanomas primarios y apuntan a una posible diana terapéutica combinada de las vías NF-kB/p53 para controlar la desviación de TAM en melanoma.

Gutiérrez-Seijo A, García-Martínez E, Barrio-Alonso C, Pareja-Malagón M, Acosta-Ocampo A, Fernández-Santos ME, Puig-Kröger A, Parra-Blanco V, Mercader E, Márquez-Rodas I, Avilés-Izquierdo JA, Samaniego R, Sánchez-Mateos P. CCL20/TNF/VEGFA Cytokine Secretory Phenotype of Tumor-Associated Macrophages Is a Negative Prognostic Factor in Cutaneous Melanoma. Cancers (Basel). 2021 Aug 5;13(16):3943. doi: 10.3390/cancers13163943. PMID: 34439098; PMCID: PMC8392234.

cancers

MDPI

Article

CCL20/TNF/VEGFA Cytokine Secretory Phenotype of Tumor-Associated Macrophages Is a Negative Prognostic Factor in Cutaneous Melanoma

Alba Gutiérrez-Seijo [1,2,†], Elena García-Martínez [1,2,†], Celia Barrio-Alonso [1,2], Miriam Pareja-Malagón [3], Alejandra Acosta-Ocampo [3], María Eugenia Fernández-Santos [3], Amaya Puig-Kröger [4], Verónica Parra-Blanco [5], Enrique Mercader [6,7], Iván Márquez-Rodas [8], José Antonio Avilés-Izquierdo [9], Rafael Samaniego [1,*,‡] and Paloma Sánchez-Mateos [2,10,*,‡]

1 Unidad de Microscopía Confocal, Instituto de Investigación Sanitaria Gregorio Marañón, 28007 Madrid, Spain; albaguti@ucm.es (A.G.-S.); egarciamartinez2@salud.madrid.org (E.G.-M.); celibarr@ucm.es (C.B.-A.)
2 Laboratorio de Inmuno-Oncología, Instituto de Investigación Sanitaria Gregorio Marañón, 28007 Madrid, Spain
3 Unidad de Producción Celular, Instituto Investigación Sanitaria Gregorio Marañón, 28007 Madrid, Spain; miriam.pareja@iisgm.com (M.P.-M.); alejandra.acosta@iisgm.com (A.A.-O.); mariuge@fibhgm.org (M.E.F.-S.)
4 Laboratorio de Inmuno-Metabolismo e Inflamación, Instituto de Investigación Sanitaria Gregorio Marañón, 28007 Madrid, Spain; amaya.puig@iisgm.com
5 Servicio de Anatomía Patológica, Hospital General Universitario Gregorio Marañón, 28007 Madrid, Spain; veronica.parra@salud.madrid.org
6 Unidad Cirugía Endocrino-Metabólica, Servicio de Cirugía General y Aparato Digestivo, Hospital General Universitario Gregorio Marañón, Calle Doctor Esquerdo 46, 28007 Madrid, Spain; enrique.mercader@salud.madrid.org
7 Laboratorio Inmunofisiología, Instituto de Investigación Sanitaria Gregorio Marañón, Calle Doctor Esquerdo 46, 28007 Madrid, Spain
8 Servicio de Oncología Médica, Hospital General Universitario Gregorio Marañón, 28007 Madrid, Spain; ivan.marquez@salud.madrid.org
9 Servicio de Dermatología, Hospital General Universitario Gregorio Marañón, 28007 Madrid, Spain; jantonio.aviles@salud.madrid.org
10 Departamento de Inmunología, Facultad de Medicina, Universidad Complutense de Madrid, 28040 Madrid, Spain
* Correspondence: confocal@hggm.es (R.S.); paloma.sanchezmateos@salud.madrid.org (P.S.-M.)
† Authors contributed equally to this work.
‡ Senior co-authors contributed equally to this work.

check for updates

Citation: Gutiérrez-Seijo, A.; García-Martínez, E.; Barrio-Alonso, C.; Pareja-Malagón, M.; Acosta-Ocampo, A.; Fernández-Santos, M.E.; Puig-Kröger, A.; Parra-Blanco, V.; Mercader, E.; Márquez-Rodas, I.; et al. CCL20/TNF/VEGFA Cytokine Secretory Phenotype of Tumor-Associated Macrophages Is a Negative Prognostic Factor in Cutaneous Melanoma. *Cancers* **2021**, *13*, 3943. https://doi.org/10.3390/cancers13163943

Academic Editor: Suzie Chen

Received: 28 June 2021
Accepted: 3 August 2021
Published: 5 August 2021

Simple Summary: Cutaneous melanoma is characterized by its heterogeneous metastatic behavior and robust biomarkers are still needed to identify those patients with increased risk for distant metastasis, to guide new adjuvant treatments. We aimed to assess the prognostic role of different features of tumor-associated macrophages (TAMs) using multicolor immunofluorescence microscopy and single-cell analysis. Rather than the number, size, or location of TAM, quantitative assessment of CCL20, TNF, and VEGFA cytokine content was associated with strong prognostic significance in primary melanoma. This novel TAM cytokine signature serves as a readout of TAM prometastatic activation and provides independent information to traditional TNM melanoma staging. In addition, we show that this particular cytokine profile is coregulated by p53 and NF-κB, suggesting that therapies targeting both pathways may modulate the prometastatic deviation of TAMs in melanoma.

Abstract: TAMs constitute a large fraction of infiltrating immune cells in melanoma tissues, but their significance for clinical outcomes remains unclear. We explored diverse TAM parameters in clinically relevant primary cutaneous melanoma samples, including density, location, size, and polarization marker expression; in addition, because cytokine production is a hallmark of macrophages function, we measured CCL20, TNF, and VEGFA intracellular cytokines by single-cell multiparametric confocal microscopy. The Kaplan–Meier method was used to analyze correlation with melanoma-specific disease-free survival and overall survival. No significant correlations with clinical parameters were observed for TAM density, morphology, or location. Significantly, higher contents of the intracellular

cytokines CCL20, TNF, and VEGFA were quantified in TAMs infiltrating metastasizing compared to non-metastasizing skin primary melanomas ($p < 0.001$). To mechanistically explore cytokine up-regulation, we performed in vitro studies with melanoma-conditioned macrophages, using RNA-seq to explore involved pathways and specific inhibitors. We show that p53 and NF-κB coregulate CCL20, TNF, and VEGFA in melanoma-conditioned macrophages. These results delineate a clinically relevant pro-oncogenic cytokine profile of TAMs with prognostic significance in primary melanomas and point to the combined therapeutic targeting of NF-kB/p53 pathways to control the deviation of TAMs in melanoma.

Keywords: CCL20; TNF; VEGFA; melanoma; metastasis; TAM; biomarker; prognostic factor

1. Introduction

Despite the significant survival benefit provided by new treatments, metastatic melanoma continues to be a life-threatening disease [1]. At the time of clinical presentation, skin melanomas are frequently excised when distant metastases are clinically undetectable and most patients are included in stage I and II (localized melanomas) or stage III (regional disease) [2]. A small proportion of cases (<4%) show distant metastases (stage IV) at the time of diagnosis of the primary tumor, whereas most metastatic cases progress from stages II-III patients during follow-up. Identification of patients with biologically aggressive melanomas is important because treatment at an earlier clinical stage improves disease-free survival but with the risk of toxicity [3]. With survival rates ranging from 63–81% in stage II and 36–63% in stage III [4], there is great variability in metastatic risk at diagnosis and robust biomarkers are currently needed. Some tumor microenvironment (TME)-derived factors or infiltrating cells, particularly immune cells, are a source of biomarkers, providing independent prognostic information for assessing metastatic risk or to predict the efficacy of new treatments [5].

Tumor-associated macrophages (TAMs) are among the most frequent immune infiltrating cells in solid tumors, displaying a variety of phenotypes and functions [6]. Single-cell analysis in lung adenocarcinoma and renal cancer has revealed an unsuspected heterogeneity of TAM phenotypes [7,8]. Diversity of macrophages was oversimplified in the M1/M2 classification, in which skewing TAMs towards M1-type may result in anti-tumor responses whereas M2 phenotype promotes tumor evolution and metastasis [9]. Characterization of macrophage polarization in human tissues is an important issue; however, due to their complexity and to differences with studies mostly performed in vitro, it has remained difficult [10]. The use of single markers as currently performed by most immunohistochemistry approaches (IHC) also prevents correct identification of M1/M2 cell subsets or differentiation from other mononuclear phagocyte populations [11]. In advanced solid tumors, TAMs are known to be preferentially M2 biased; however, cutaneous melanomas are generally non-advanced primary tumors at diagnosis and M1/M2 subsets remain to be properly addressed in large patient groups [12]. Several studies have evaluated TAM number/density as prognostic factors for stage I/II melanomas with opposite results. Jensen et al. [13], using the general marker of human macrophages CD68 and CD163 as a single M2 marker, showed that macrophage infiltration at the invasive front was an independent predictor of poor survival. Subsequently, other large-scale studies did not corroborate the correlation between CD68 positive TAM number and melanoma survival [14,15].

Complex paracrine interactions among tumor cells and a variety of non-tumoral components and infiltrating cells take place in the TME and impact tumor biology [16]. We previously reported that in human melanoma the chemokine axis CCR6/CCL20 is involved in a cooperative paracrine loop between CCR6 expressing tumor cells and CCL20 secreted by nontumoral cells in the stroma [17]. Importantly, we showed that stromal CCL20 predicted poor survival in a cohort of 40 primary melanoma patients and identified TAMs as the main stromal source of CCL20 in melanoma tissues. Thus, we hypothesize

that the CCL20 secretory TAM phenotype might be associated with melanoma progression and could be used for an accurate prognosis of metastatic risk in skin melanoma. In the current study, we measured diverse characteristics of tissue TAMs, including CCL20 and other cytokines by multicolor immunofluorescence and single-cell analysis, and correlated them with the clinical outcome of cutaneous melanoma patients.

2. Materials and Methods

2.1. Study Cohorts and Selection Criteria

All clinical data and patient samples were collected following approval by the Hospital Gregorio Marañón ethics committee and informed consent was obtained for each patient. A frozen-preserved collection of skin melanomas (punch biopsies not compromising patient diagnosis) and corresponding patient data were previously described [17] (Table S1). We also retrieved 83 formalin-fixed and paraffin-embedded (FFPE) primary cutaneous melanomas; with a median patient follow-up of 77 ± 47 months, >2 mm Breslow thickness, excised between 1998 and 2015 in our institution; with either clinically aggressive disease developing distant metastasis or lymph node recurrence during follow-up (51 metastasizing primary melanomas, with 33/51 melanoma-related and 2/51 non-related deaths) or matched skin melanoma samples from patients who were disease-free for at least 10 years of follow-up (32 non-metastasizing primary melanomas). Pathological AJCC staging II-IV assessment was obtained by sentinel lymph node biopsy and distant metastasis evaluation by computed tomography at the time of diagnosis. Follow-up studies were performed every 6 months for 5 years and then annually up to 10 years unless there was an earlier documented metastatic event (regional or distant metastasis). Patients were selected because they were representative of opposite clinical outcomes during follow-up, therefore, primary tumors were thicker (median and mean, 3.9 mm and 4.9 mm, respectively) than in a population-based registry data [18]. Metastasizing and non-metastasizing primary tumors had comparable Breslow thickness (mean, 5.3 mm and 4.2 mm, respectively; Mann–Whitney, $p = 0.47$). Six patients with immediate recurrence after the first diagnosis were excluded for disease-free survival (DFS) but not for overall survival (OS) (Table S1).

2.2. Multicolor Fluorescence Confocal Microscopy

FFPE sections were de-paraffinized, rehydrated, and unmasked by steaming in 10 mM sodium citrate buffer pH 6.0 (Dako) for 6 min. Slides were blocked with 5 µg/mL human immunoglobulins (Ig) solved in blocking serum-free medium (Dako) for 30 min, and then sequentially incubated with 5–10 µg/mL primary antibodies specific for CCL20 (Abcam ab9829, polyclonal rabbit IgG), TNFα (Abcam ab1793, clone 52B83, mouse IgG1), VEGFA (Antibodies online, ABIN191867, rabbit IgG), CD115 (R&D AF329, polyclonal Goat IgG), CD163 (Bio-Rad MCA1853T, clone EDHu-1, mouse IgG1) and/or CD68 (Dako, clone PG-M1, mouse IgG3) in phosphate buffer solution (PBS) containing 10% blocking medium overnight at 4 °C, and then proper fluorescent secondary antibodies (Jackson-Immunoresearch, Invitrogen) for 1 h at room temperature. Intermediate washes were performed in agitation and by immersion in PBS containing 0.05% Tween-20. Samples mounted with fluorescent mounting medium (Dako) were imaged with a Leica SPE confocal microscope using a glycerol-immersion ACS APO 20×/NA 0.60 objective. Cytokines used in immunocompetition assays were purchased from Immunotools.

Single-cell quantification was performed in 3–5 20× fields, as previously described [17,19,20]. For proper TAM segmentation, whole-cell area staining is necessary to define macrophages contour, which is best achieved by CD163 staining in frozen samples fixed with acetone, and CD68 labeling in formalin-fixed paraffin-embedded sections. Mean fluorescence intensity (MFI) of proteins of interest was obtained at manually depicted TC nests or at each segmented TAM using the 'analyze particle' plugging of FIJI software. For TAM density, 3–5 fields were quantified, discriminating two different regions. Intratumoral regions corresponded to identifiable tumor nests, whereas stromal regions were characterized by elongated DAPI stained nuclei, including stromal bundles wider

than 30 µm and peritumoral areas up to 300 µm far from melanoma cells. Only regions clearly differentiable were included in the study.

2.3. Cell Isolation from Human Melanomas

Biopsies from stage IV melanoma patients were homogenized and digested into single-cell suspensions (Tumor Dissociation Kit, Miltenyi, Bergisch Gladbach, Germany). TAMs and tumor-infiltrating lymphocytes were purified by magnetic cell sorting using CD14 and CD3 microbeads, respectively. The remaining negatively selected cells were considered as 'TCs' [17,19].

2.4. Monocyte Isolation and Cell Culture

Peripheral blood mononuclear cells (PBMCs) were isolated from patients or buffy-coats from healthy donors over a ficoll gradient (Lymphocytes Isolation Solution, Rafer). Monocytes were purified by magnetic cell sorting using anti-CD14 tagged microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany). For in vitro generation of macrophages, monocytes were cultured at 0.5×10^6/mL for 7 days containing GM-CSF (10 ng/mL or 1000 U/mL, Immunotools) or M-CSF (10 ng/mL, Immunotools) to generate MP-GM or MP-M macrophages, respectively. Cytokines were added every two days. All cells, including melanoma cell lines BLM and A375 [21] were cultured in RMPI-1640 medium (Gibco, Waltham, MA, USA) supplemented with 10% fetal calf serum (FCS, Sigma, Burlington, MA, USA).

2.5. In Vitro Measurements

Melanoma cells were cocultured with macrophages at 1:2 ratios (melanoma/macrophage). After 24 and 72 h, supernatants were collected for secreted proteins assessment (CCL20, TNF, and VEGFA ELISAs, R&D Systems), and magnetically separated cells were processed for quantitative real-time PCR (qPCR) analyses. When specified, cultures contained 10 µM BAY11-7082 (NF-κB-inhibitor, ChemCruz) and/or 50 µM Pifithrin-α (p53-inhibitor, Sigma).

For qPCRs, oligonucleotides (CCL20, S 5′ gctgctttgatgtcagtgct; AS 5′ gcagtcaaagttgcttgctg; TNF, S 5′ cagcctcttctccttcctgat; AS 5′ gccagagggctgattagaga; VEGFA, S: 5′ gcagcttgagttaaacgaacg; AS: 5′ ggttcccgaaaccctgag) were designed according to the Roche software for qPCR. Total RNA was extracted (NucleoSpin RNA-purification kit, Macherey Nagel, Düren, Germany) and retrotranscribed cDNA quantified using the Universal Human Probe Roche library (Roche-Diagnosis, Basel, Switzerland). Assays were made in triplicate and normalized to TBP and/or HPRT1 expression (ΔΔCT method). For RNAseq and Gene Set Enrichment Analysis (GSEA), total RNA was isolated from three independent preparations and processed at BGI (https://www.bgi.com, accessed on 4 August 2021), where library preparation, fragmentation, and sequencing were performed using the DNBseq platform. An average of 4.39 Gb bases were generated per sample and, after filtering, clean reads were mapped to the reference (UCSC Genome assembly hg38) using Bowtie2 (average mapping ratio 95.49%) [22]. Gene expression levels were calculated by using the RSEM software package [23]. Differential gene expression was assessed by using DEseq2 algorithms using the parameters Fold change >2 and adjusted p-value < 0.05. For gene set enrichment analysis (GSEA) (http://www.broad.mit.edu/gsea/, accessed on 4 August 2021), the gene sets available at the website [24], as well as the "HALLMARK_P53_PATHWAY" and "HALLMARK_TNFA_SIGNALING_VIA_NFKB" gene sets, that contain the top genes involved in p53 and NF-kB pathways respectively, were used [25]. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus [26] and are accessible through GEO Series accession number GSE171277.

2.6. Statistical Analyses

Censured Kaplan-Meier curves were used to analyze the correlation with patient disease-free and overall survival, and the Cox-regression method (univariate and multivariate) to identify independent prognostic variables. Mann-Whitney t-test was used to

evaluate the association with clinicopathological features. The paired t-test, Spearman R correlation, and Log-rank analyses have also been used in this study (Graph-Pad software, San Diego, CA, USA), as indicated. $p < 0.05$ was considered statistically significant.

3. Results

Motivated by the idea of identifying prognostic biomarkers derived from the TME, we sought to identify and quantify diverse TAM subsets/characteristics in two independent collections of primary melanoma samples with clinical information and the survival status of the patients. Table S1 lists the clinicopathological characteristics of 40 cryopreserved primary melanomas collected in a prospective patient cohort, and 83 FFPE samples retrieved from the archive, as described in Material and Methods. Primary melanomas were classified as non-metastasizing or metastasizing regarding subsequent development of metastasis during patient follow-up of 5 and 10 years in each cohort, respectively.

We first analyzed macrophage subsets in cryopreserved samples, using triple-color labeling with CD11c, CD209, and CD163, to quantify cells with macrophage morphology in normal skin, skin melanoma metastases, and primary melanoma tumors (non-metastasizing and metastasizing) (Figure 1A) [17]. CD163, well expressed by most tissue macrophages [11], was used to gate macrophages and to measure CD11c (M1 marker) and CD209 (M2 marker) expression levels in gated $CD163^+$ cells (Figure 1A). Accordingly, subsets were defined as follows: a $CD209^+CD11c^-$ M2 subset, which was the only group detected in normal skin (depicted in red in control skin dot-plot), $CD209^-CD11c^+$ M1 subset, which appeared as a new population in both metastases and primary melanomas, and $CD209^+CD11c^+$ (mixed population) (Figure 1A,B). Comparative analysis of 24 non-metastasizing versus 16 metastasizing primary tumors showed no difference in $CD209^-CD11c^+$ (M1-like) and $CD209^+CD11c^+$ (M1/M2 mixed) subsets, with a tendency to less $CD209^+CD11c^-$ (M2-like) macrophages in metastasizing primary melanomas ($p = 0.058$) (Figure 1B), consistent with our previous pilot study with 5 non-metastasizing versus 5 metastasizing cases [17]. We next quantified TAM density, which is a common parameter used to test the clinical relevance of immune cell populations, and TAM size, which was reported to correlate with functional diversity and polarity and may provide prognostic significance [27]. However, we did not find significant differences in TAM density or size between non-metastasizing and metastasizing primary tumors (Figure 1C).

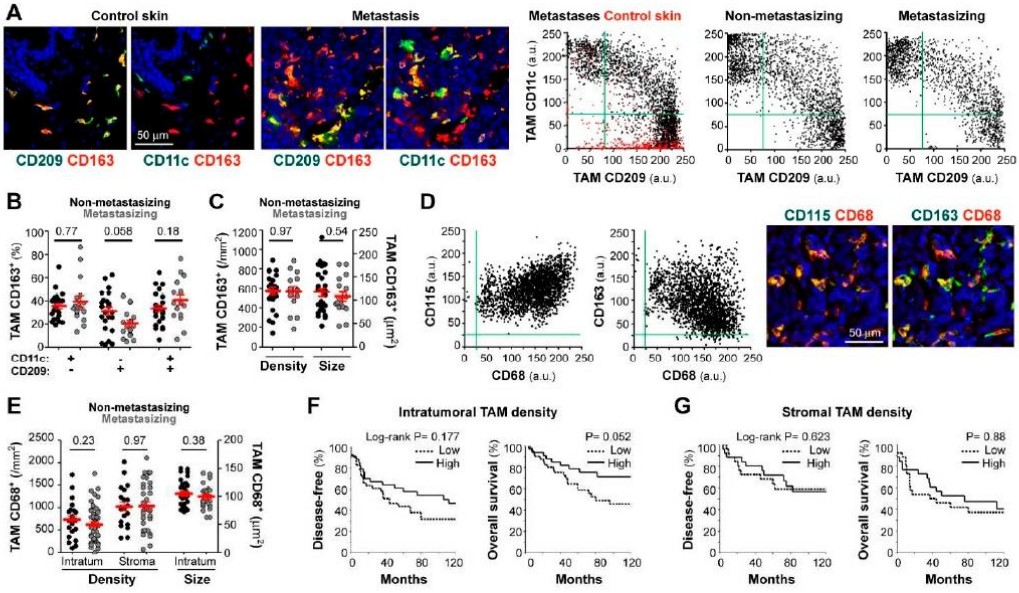


Figure 1. Correlation between patient evolution and TAM density, size, and polarization state. Confocal microscopy screening of two independent and differently preserved primary melanoma collections: frozen (stage I-III; (**A**–**C**)) and FFPE (stage II-IV; **D**–**G**). (**A**) Frozen tissues co-stained for CD163 (red) and CD11c or CD209 (green), as indicated. Dot-plots show single-cell MFI (arbitrary units, a.u.) for CD11c and CD209 proteins simultaneously quantified at CD163$^+$ TAMs in control skin (n = 3, red dots), skin metastases (n = 6), non-metastasizing (n = 24) and metastasizing (n = 11) primary melanomas. (**B**) Relative percentages of polarized TAMs through the whole cryopreserved primary collection (n = 40). (**C**) Average density (by mm^2) and size (µm^2) of CD163$^+$ TAMs quantified through intratumoral and/or peritumoral regions of primary melanomas (n = 40). (**D**) Paraffin-embedded melanoma co-stained for pan-macrophage markers CD68 (red) and CD163 or CD115 (green), as indicated. Dot-plots show single-cell expression of CD68 vs. CD115 or CD163 proteins quantified in either CD68$^+$, CD163$^+$ and/or CD115$^+$ TAMs (n = 3 non-metastasizing and 3 metastasizing primary melanomas). (**E**) CD68$^+$ TAM density quantified through intratumoral (n = 83) and stromal (n = 54) areas of primary melanomas. TAM size at intratumoral regions (n = 83), are shown. (**F**,**G**) Correlation between patient evolution and CD68$^+$ TAM density at intratumoral (**F**) and stromal (**G**) regions. Disease-free and overall survival 10-year Kaplan–Meier curves are shown. Cut-off values were calculated from ROC curves to classify as 'low' or 'high' density. p values are shown (Log-rank). Mann-Whitney statistical analysis was used to compare non-metastasizing vs. metastasizing melanomas in (**B**,**C**,**E**) panels. Scale bars, 50 µm.

Because harvesting primary melanoma tissues for cryopreservation is only feasible when sufficient tumor is available and requires close coordination between clinicians, we set up conditions for multicolor staining of macrophage markers in routine FFPE diagnostic material. Triple staining with CD68, CD115 (macrophage growth factor receptor, CSF1R), and CD163 demonstrated co-expression by most cells with macrophage morphology (Figure 1D). There was negative correlation between CD68 and CD163 mean fluorescence intensity (MFI) (R= −0.36, n = 6, $p < 0.001$); however, neither CD163 nor CD68 nor CD115 defined separated M1/M2 macrophage subsets, indicating that they are pan-macrophage markers in melanoma. For image quantification, we chose CD68 for macrophage segmentation because very few CD68$^+$ cells were negative for CD115 or CD163, indicating that very few cells other than macrophages were CD68$^+$ in melanoma tissues. We quantified TAM density in full-face FFPE primary melanoma sections, finding no significant differences between 32 non-metastasizing and 51 metastasizing primary tumors, regarding stromal or intratumoral TAM density (Figure 1E). Furthermore, the size of intratumoral TAMs was not different between the two clinically divergent groups

(Figure 1E). We next defined low and high TAM density (in tumor nests and stromal areas independently) using the best cut-off value extrapolated from the ROC curve (receiver operating characteristic) and calculated 10-years DFS and OS Kaplan-Meier survival curves (Figure 1F,G, for intratumoral and stromal TAMs, respectively), finding a tendency of high intratumoral TAM density towards a better patient survival (Figure 1F, $p = 0.052$).

We previously identified co-expression of CCL20, TNF, and VEGFA cytokines by tissue TAMs in an exploratory set of 7 metastasizing melanomas [17]. Now, we tested the hypothesis that this distinctive prometastatic TAM secretory phenotype can be identified in routine FFPE samples, to be clinically applicable for patient profiling. First, we established conditions to quantify by multicolor fluorescent-microscopy the content of CCL20, TNF, and VEGFA in $CD68^+$ cells in FFPE melanoma tissues. To validate the staining specificity, we preincubated the anti-cytokine antibodies with an excess of each cytokine, confirming that staining was specifically blocked on FFPE melanoma tissues (Supplementary Figure S1). Figure 2A shows representative images of $CD68^+$ TAMs merged with CCL20, TNF, or VEGFA of two primary melanoma samples representative of low and high cytokine expression profiles. Dot plots in Figure 2B represent quantification at the single-cell level of VEGFA and TNF MFI in accumulated TAMs from $n = 11$ non-metastasizing melanomas compared with $n = 22$ metastasizing primary melanomas, showing higher TNF/VEGFA expression by $CD68^+$ TAMs in clinically aggressive melanomas. Correlations between TNF and VEGFA MFI levels ($R = 0.69$, $n = 22$, $p < 0.001$) or TNF and CCL20 ($R = 0.62$, $n = 10$, $p < 0.001$) by TAMs were found among cases of metastasizing tumors, indicating a positive relationship among these protumoral cytokines. To assess the cytokine expression profile of primary tissue cells with a different methodology, we purified TAMs and tumor cells from 4 thick metastasizing primaries (stage IV patients at diagnosis) to quantify mRNA expression, showing active gene expression of TNF, CCL20, and VEGFA by TAMs compared to peripheral blood monocytes isolated from these patients. In contrast, remaining cancer cells transcribed less *CCL20*, *TNFA*, and *VEGFA* than TAMs (Figure 2C). Next, we used the Kaplan–Meier method to assess the clinical relevance of quantifying the MFI expression level of CCL20, TNF, and VEGFA cytokines by TAMs in FFPE melanoma tissues from 83 primary melanoma patients (Figure 2D,E). Patients were stratified as 'high' or 'low', using the cell-specific median MFI value for each cytokine as the cut-off point. High TAM-CCL20 or TAM-TNF content correlated with shorter DFS and OS (log-rank test, $p \leq 0.01$), whereas high TAM-VEGFA expression correlated only with shorter DFS ($p = 0.005$). CCL20 and TNF were not expressed by cancer cells and no correlation was found between cancer cell-VEGFA content and DFS or OS ($p = 0.19$and $p = 0.63$, respectively) (Table 1 and Table S2). To determine whether TAM-specific quantification of CCL20, TNF, or VEGFA were independent prognostic factors, we performed a multivariate regression analysis including gender, age, location, histologic type, ulceration, Breslow, and stage parameters (Table 1). This analysis showed that TAM-specific CCL20 and TNF expression levels were independent prognostic factors for DFS ($p < 0.001$) and OS ($p = 0.004$ and $p = 0.001$, respectively) in this cohort. Whereas TAM-VEGFA content was an independent predictor of DFS ($p < 0.001$) but did not reach significance for OS. Finally, we analyzed the prognostic value of the CCL20/TNF/VEGFA combined secretory TAM phenotype, showing its great potential in the prediction of DFS and OS of primary melanoma patients ($p < 0.0001$ and $p = 0.0004$, respectively) (Figure 2F). Consequently, we validated our previous pilot study in a large and independent patient cohort, using standard FFPE tissue sections instead of cryosections, and confirmed the role of CCL20, TNF, and VEGFA expression by TAMs in predicting clinical behavior of primary cutaneous melanoma patients. Our results highlight the role of TAMs in biologically aggressive primary melanomas, suggesting that prometastatic TAMs are characterized by higher secretion of CCL20/TNF/VEGFA cytokines.

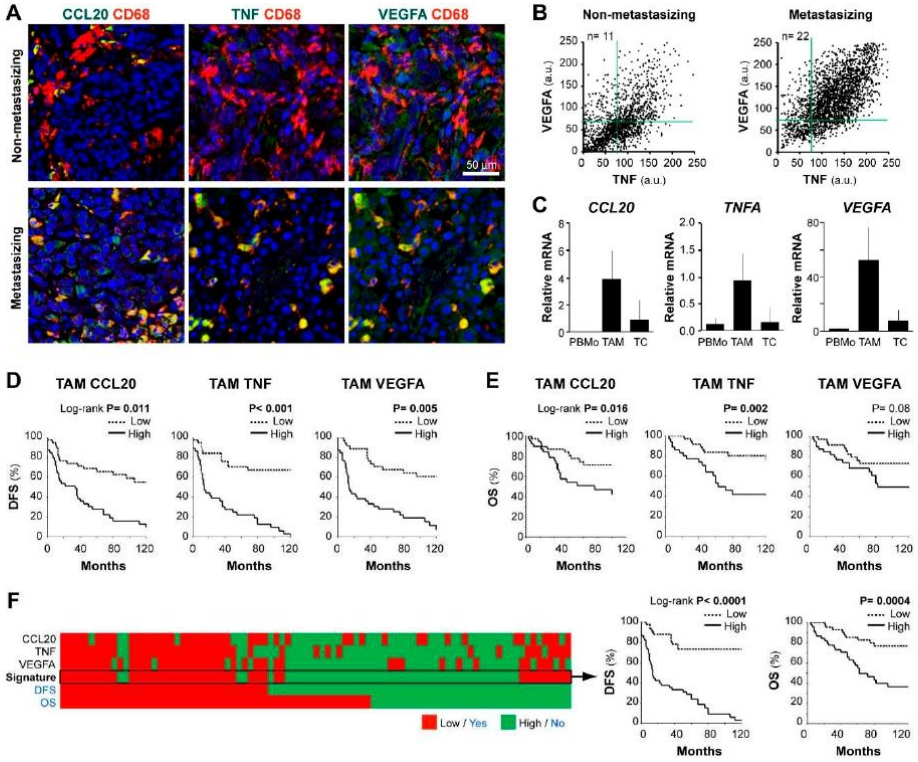


Figure 2. CCL20, TNF, and VEGFA expression in human melanoma TAMs and correlation with patient survival. (**A**) FFPE human melanoma samples co-stained for CD68 (TAM marker, red) and CCL20, TNF, or VEGFA (protumoral markers, green), as indicated. Representative images of non-metastasizing and metastasizing primary melanomas are shown. Scale bar, 50 µm. (**B**) Dot-plots showing CD68+TAMs from non-metastasizing (n = 11 samples; 1100 TAMs) and metastasizing (n = 22 samples; 2200 TAMs) primary melanomas. (**C**) Expression of *CCL20*, *TNFA*, and *VEGFA* mRNA (relative to average *TBP/HPRT1*) in freshly isolated peripheral blood monocytes $CD14^+$ (PBMo), $CD14^+$ TAMs, and negatively selected cells ($CD14^-CD3^-$) from three-stage IV primary melanoma patients. (**D**,**E**) Correlation between patient evolution and TAM expression of CCL20, TNF, and VEGFA proteins. Disease-free (**D**) and overall (**E**) survival 10-year Kaplan–Meier curves are shown. Median values of the 83 primary melanomas were used to classify as 'low' or 'high' expressing samples. p values are shown (Log-rank). (**F**) Kaplan–Meier curves using CCL20/TNF/VEGFA as a whole signature (classified as 'high' or 'low' by majority: ≥2/3). DFS, disease-free survival. OS, overall survival.

Table 1. Univariate and multivariate Cox regression analyses for 10-year disease-free and overall survival (FFPE cohort).

Univariate	**Disease-Free Survival**			**Overall Survival**		
Gender (F vs. M)	1.602	0.91–2.81	0.100	1.168	0.55–2.47	0.685
Age (years)	1.003	0.99–1.02	0.704	1.007	0.98–1.03	0.574
Location (H/L vs. T)	1.452	0.74–2.85	0.278	0.895	0.40–1.99	0.785
Subtype (nod vs. others)	0.831	0.46–1.52	0.547	1.305	0.60–2.82	0.498
Ulceration (yes vs. no)	1.354	0.76–2.41	0.301	2.582	1.15–5.82	0.022
Breslow (mm)	1.130	1.07–1.20	<0.001	1.124	1.04–1.21	0.002
Stage (II vs. III–IV)	1.865	1.19–2.92	0.006	1.793	0.74–4.33	0.194
TAM-CCL20 (MFI, each 10 a.u.)	1.195	1.10–1.29	<0.001	1.164	1.05–1.29	0.003
TAM-TNF (MFI, each 10 a.u.)	1.295	1.18–1.42	<0.001	1.250	1.11–1.41	0.000
TAM-VEGFA (MFI, each 10 a.u.)	1.185	1.11–1.26	<0.001	1.051	0.96–1.15	0.264
TC-VEGFA (MFI, each 10 a.u.)	1.050	0.98–1.13	0.189	1.026	0.92–1.14	0.629
Multivariate	**HR**	**95% CI**	***p***	**HR**	**95% CI**	***p***
Gender (F vs. M)	1.019	0.56–1.87	0.951	0.612	0.25–1.47	0.274
Age (years)	0.999	0.98–1.02	0.861	1.010	0.99–1.03	0.387
Breslow (mm)	1.164	1.09–1.25	<0.001	1.173	1.06–1.30	0.002
Stage (II vs. III–IV)	0.716	0.33–1.53	0.390	0.596	0.17–2.10	0.421
TAM-CCL20 (MFI, each 10 a.u.)	1.244	1.12–1.38	<0.001	1.260	1.08–1.47	0.004
Gender (F vs. M)	1.245	0.71–2.20	0.449	0.666	0.27–1.64	0.376
Age (years)	0.996	0.98–1.01	0.692	1.005	0.98–1.03	0.715
Breslow (mm)	1.115	1.05–1.18	<0.001	1.123	1.03–1.22	0.008
Stage (II vs. III–IV)	1.401	0.90–2.19	0.138	1.013	0.35–2.92	0.982
TAM-TNF (MFI, each 10 a.u.)	1.304	1.19–1.43	<0.001	1.255	1.09–1.44	0.001
Gender (F vs. M)	1.103	0.61–2.00	0.747	0.811	0.34–1.91	0.632
Age (years)	1.005	0.99–1.02	0.577	1.008	0.98–1.03	0.550
Breslow (mm)	1.138	1.06–1.22	<0.001	1.124	1.03–1.23	0.008
Stage (II vs. III–IV)	1.122	0.63–1.99	0.694	1.230	0.46–3.32	0.683
TAM-VEGFA (MFI, each 10 a.u.)	1.188	1.11–1.27	<0.001	1.040	0.95–1.14	0.404

To search for mechanisms underlying the CCL20/TNF/VEGFA secretory TAM phenotype associated with aggressive primary melanomas, and due to limited access to patient TAMs, we prepared tumor-conditioned macrophages. We previously showed that both pro-inflammatory (GM-CSF) and anti-inflammatory (M-CSF) human macrophages (MP-GM and MP-M, respectively) co-cultured with melanoma metastatic cell lines (BLM and A375), displayed a distinctive CCL20 mRNA up-regulation and secreted large amounts of CCL20 protein [17]. Similarly, we now observed up-regulation of TNF and VEGFA mRNA expression by melanoma-conditioned MP-GM(BLM) and, to a lesser amount, by MP-M(BLM), which were separated from BLM melanoma cells after co-culture (Figure 3A). Melanoma BLM cells were also analyzed after co-culture, showing no expression of CCL20 and low induction of *TNF* mRNA, whereas *VEGFA* mRNA was basally expressed by BLM cells. To get insights into the molecular programs activated by melanoma-conditioning of MP, we performed RNA sequencing on MP-GM and MP-M with and without co-culturing with BLM cells. 1036 genes were found significantly differentially expressed in melanoma-conditioned MP-GM(BLM) versus MP-GM, and 1677 genes in melanoma conditioned MP-M versus MP-M(BLM) (adjusted p-value < 0.05; log2 fold change >2) (Figure 3B). Pathway analysis identified a number of pathways related to TNF_NF-κB and p53 signaling in the melanoma-conditioned MPs (Figure 3B).

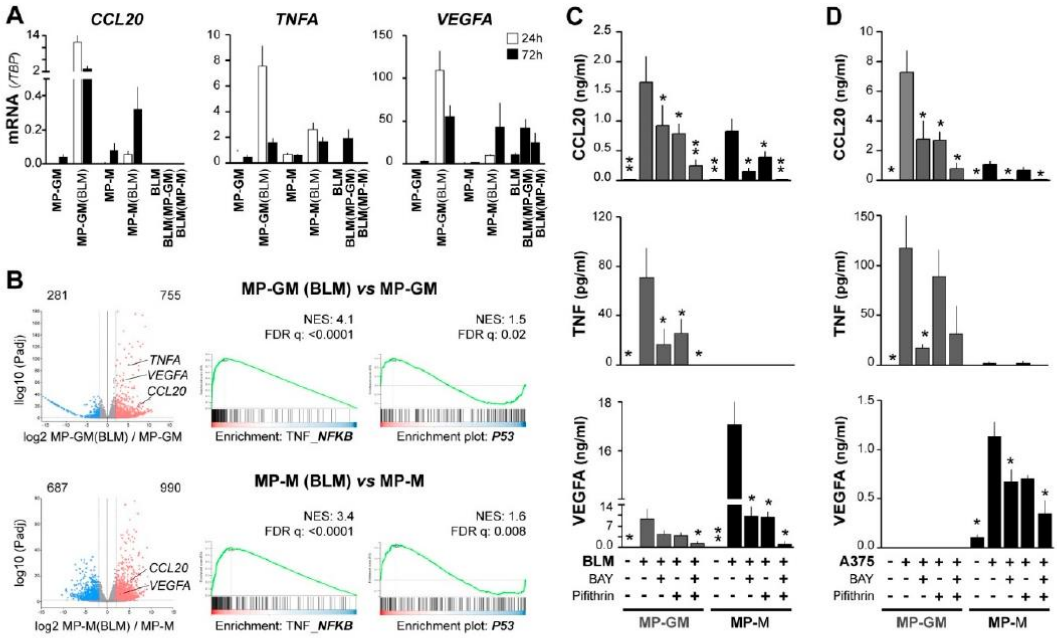


Figure 3. NF-κB and p53 signaling pathways synergically contribute to CCL20, TNF, and VEGFA upregulation in macrophages conditioned by melanoma cells. (**A**) Macrophages differentiated with GM-CSF or M-CSF (MP-GM and MP-M, respectively) were cocultured with BLM cells for 24 h or 72 h, as specified, and then separated with anti-CD14-coated beads. Mutually conditioned cells were measured for *CCL20*, *TNF*, and *VEGFA* mRNA expression relative to *TBP* (n = 3–5 donors). (**B**) Volcano plots showing DEG scores for 24h BLM-conditioned macrophages vs. unconditioned macrophages, as well as GSEA analyses showing normalized enrichment scores and false discovery rates (n = 3). (**C**,**D**) Conditioned media assessed for CCL20, TNF, and VEGFA protein secretion. As in A, differentiated macrophages were cocultured with BLM (**C**) or A375 (**D**) melanoma cells for 72 h in the absence or presence of 10 μM BAY11-7082 (NF-κB inhibitor) and/or 50 μM Pifithrin-α (p53 inhibitor), as indicated (n = 5–10). Asterisks denote statistically significant differences relative to melanoma/macrophage condition cocultured without inhibitors (paired t-test; * $p < 0.05$; ** $p < 0.01$).

To explore the effect of targeting these pathways during co-cultures of MPs (GM and M) with melanoma cells, we used BLM and A375 (which do not express VEGFA basally) metastatic melanoma cell lines, which similarly induced secretion of the three cytokines in the co-cultures (Figure 3C,D). However, there were differences in the cytokine profile up-regulated regarding the MP polarization profile; MP-GM/melanoma produced more TNF and CCL20, whereas MP-M/melanoma produced more VEGFA. Next, we used BAY 11-7082 to inhibit the NF-κB signaling cascade and the p53 inhibitor pifithrin and analyzed secretion of CCL20, TNF, and VEGFA proteins in the supernatants; getting additive inhibitory effects in the secretion of the three cytokines. This data indicate that macrophage-melanoma interactions induced both signaling pathways, NF-κB and p53 activation, which may contribute to up-regulate the specific cytokine secretory phenotype of TAMs, regarding their previous M1/M2 polarization heterogeneity.

4. Discussion

Because TAMs are a very heterogeneous population of immune cells, which has been reported to be associated with either anti-tumoral or protumoral functions, assessment of their molecular or functional variety may provide new biomarkers for patient prognosis or to predict treatment responses [6,10]. Accurate prognosis in melanoma is particularly important to identify those patients who might benefit from adjuvant treatment. In the

present study, we have quantitatively evaluated conventional parameters like density, morphology, stroma/tumor nest distribution, as well as more in the deep characterization of TAM polarization and cytokine content. In clinically relevant primary melanoma patient cohorts, we did not find a significant association of TAM density, size, and location, nor CD11c/CD209 subsets with DFS or OS. In prior work, our group identified a particular secretory phenotype of TAMs isolated from melanoma tissues [17]. Melanoma TAMs were also characterized in situ by their high content of CCL20, TNF, and VEGFA by multicolor immunofluorescence in cryopreserved samples; we now show that this particular cytokine profile may be detected and quantified in diagnostic FFPE. Importantly, the high content of CCL20/TNF/VEGFA is strongly associated with a worse prognosis in primary melanoma patients.

Nowadays it is well recognized that the interaction of the cancer cells with other cells or factors in the TME has an impact on tumor biology [16]. A deep analysis of TME may provide clues to understanding these complex relationships and, ultimately, will be of clinical utility for the establishment of novel prognostic tools or therapeutic targets. To this end, we previously used multicolor immunofluorescence microscopy to study in situ diverse immune and stromal components, as well as the expression of chemokines and their receptors in the TME of human melanoma. This non-destructive tissue methodology allowed us to study the spatial distribution of diverse cells, location of specific proteins within cells or tissue areas, and quantification of their relative level of expression. Human tissue studies commonly use a chromogenic IHC approach to evaluate specific proteins or cell markers in situ; however, only single or double-labeling are suitable. Fluorescent IHC and spectral microscopy have the advantage of multiple marker detection; accordingly, it was used to analyze immune infiltrates in primary melanomas, showing that combination assessment of $CD8^+$ lymphocytes/$CD68^+$ macrophages ratio in the stroma correlated with shortened survival [28]. We used a similar approach, combined with confocal microscopy and image processing, to quantify at the cellular level diverse TAM characteristics in melanoma. Multiple labeling is of particular relevance when evaluating heterogeneous cells like macrophages, which are known to share markers with other myeloid cells or to express molecules in relation to their functional polarization. As an example, CD11c, a marker traditionally associated with dendritic cells (DCs), is also expressed by M1 macrophages [29]. In previous studies, we established that TAMs are the most abundant type of myeloid cells in melanoma tissues, as compared with other mononuclear ($CCR2^+$ monocytes or $CD1c^+$ myeloid DCs) and polymorphonuclear phagocytes [17,19]. In this study, we set up conditions for multilabeling and quantitative image analysis of TAM markers in both cryopreserved and paraffin-embedded tissues, which are more convenient for clinical utility. First, as bona fide pan-macrophage markers, we showed that CD163, CD68, and CD115 were co-expressed by most cells with macrophage morphology. CD163 did not define any separated subset of TAMs, which is in agreement with its co-expression with both M1 and M2 markers in Th1 and Th2-predominant pathologies [11]. Definition of TAM subpopulations was better achieved by triple staining with a pan-macrophage marker to gate macrophages, and quantitative assessment of the level of expression of CD11c and CD209. Thus, dot-plot analysis of CD11c versus CD209 expression at the single-cell level showed that in control skin most macrophages were $CD209^+CD11c^-$ cells, which may represent tissue-resident macrophages [30]. By contrast, melanoma tissues contained a novel $CD11c^+CD209^-$ subset, maybe representing incoming proinflammatory macrophages, and $CD11c^+CD209^+$ mixed cells. However, no major differences were observed between clinically divergent groups regarding CD11c/CD209 subsets or other TAM parameters as density, morphology, or location.

Cytokine production by macrophages is a hallmark of their polarized function [9]; M1 release large amounts of proinflammatory cytokines (such as IL-12, IL-23, and TNF), whereas M2 are characterized by high expression of IL-10 and proangiogenic growth factors such as IL-8, VEGFA, and VEGFC. Interestingly, CCL20 secreting TAMs were induced via IL-6-regulated macrophage polarization in a mouse model of colon cancer; in which,

CCL20 promoted cancer progression by recruiting $CCR6^+$ lymphocytes [31]. Macrophage derived-TNF was identified as a crucial melanoma growth factor that contributed to resistance to MAPK pathway inhibitory treatments in a mouse model of $Braf^{V600E}$ mutated melanoma [32]. We had previously identified the expression of CCL20/TNF/VEGFA cytokines by TAMs in a pilot study with cryopreserved human melanoma tissues [17]. Because FFPE tissues are the current method for patient sample preservation, we validated our multiple-labeling methodology to analyze TAM cytokine signature in 83 FFPE samples from a second patient cohort. Importantly, CCL20/TNF/VEGFA cytokine production by TAMs correlated with poor patient survival. Using monocyte-derived macrophages, with either GM-CSF or with M-CSF, which are known to prime towards M1 or M2 phenotypes, we produced in vitro melanoma-conditioned macrophages expressing CCL20 and TNF (M1-primed) or VEGFA (M2-primed). This in vitro culture system of macrophages with melanoma cells, allowed us to explore the pathways underlying the CCL20/TNF/VEGFA cytokine secretory phenotype of TAMs, showing that p53 and NF-κB coregulate tumor-conditioned macrophages. It is known that NF-κB activation drives M2 polarization of TAMs [33] and its inhibition re-educates TAMs towards an anti-tumoral phenotype [34]. In accordance with our results, dual p53 and NF-κB activation in macrophages were described to up-regulate IL6, TNF, and CCL20 [35]. These results identify a unique p53/NF-kB pathway in protumoral TAMs that could be targetable to re-educate TAMs to block their protumoral functions.

5. Conclusions

Our studies show that CCL20/TNF/VEGFA cytokine production by TAMs is a hallmark of their protumoral deviation. Assessment of TAM cytokine profile in situ by multicolor staining of FFPE tissues is feasible and may be used as a new prognostic marker in cutaneous melanoma. In addition, we identify p53 and NF-κB as the main pathways driving the skewed production of CCL20/TNF/VEGFA cytokines by TAMs, pointing to the development of new therapeutic targets to re-educate this particular deviation of TAMs and to promote their anti-tumoral function.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/cancers13163943/s1, Figure S1: Validation of staining specificity, Table S1: Frozen and paraffin-embedded primary melanoma collections, clinicopathological features, Table S2: Association of evaluated prognostic markers with clinicopathological features (Mann-Whitney).

Author Contributions: Investigation, methodology, project administration, validation: A.G.-S., E.G.-M., C.B.-A. and R.S.; data curation, resources: M.P.-M., A.A.-O., M.E.F.-S., A.P.-K., V.P.-B., E.M., I.M.-R., J.A.A.-I. and P.S.-M.; conceptualization, funding acquisition, supervision, writing original draft: R.S. and P.S.-M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Ministry of Economy and Competitiveness ISCIII-FIS grant PI17/01324 (P.S.-M., R.S.) co-financed by ERDF/FEDER Funds from the European Commission, "A way of making Europe"; and by Cancer Research UK, FCAECC [GCB15152947MELE] and AIRC under the Accelerator Award Program. A.G.-S. and C.B.-A. were financed by the Comunidad de Madrid YEI-program.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of HGUGM.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Available at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE171277 (accessed on 4 August 2021).

Acknowledgments: We thank Julia Villarejo and José María Bellón for their expert technical assistance.

Conflicts of Interest: The authors declare no conflict of interest, but IMR who declares the following potential conflict of interest as an advisory of Amgen, BMS, GSK, Novartis, MSD, Roche, Celgene,

Pierre Fabre, Highlight Therapeutics (formerly Bioncotech), Regeneron, Sanofi, Merck Serono, Astra Zeneca, BiolineRx.

References

1. Curti, B.D.; Faries, M.B. Recent Advances in the Treatment of Melanoma. *N. Engl. J. Med.* **2021**, *384*, 2229–2240. [CrossRef] [PubMed]
2. Balch, C.M.; Gershenwald, J.E.; Soong, S.J.; Thompson, J.F.; Atkins, M.B.; Byrd, D.R.; Buzaid, A.C.; Cochran, A.J.; Coit, D.G.; Ding, S.; et al. Final version of 2009 AJCC melanoma staging and classification. *J. Clin. Oncol.* **2009**, *27*, 6199–6206. [CrossRef]
3. Schuchter, L.M. Adjuvant Melanoma Therapy—Head-Spinning Progress. *N. Engl. J. Med.* **2017**, *377*, 1888–1890. [CrossRef]
4. Miller, R.; Walker, S.; Shui, I.; Brandtmuller, A.; Cadwell, K.; Scherrer, E. Epidemiology and survival outcomes in stages II and III cutaneous melanoma: A systematic review. *Melanoma Manag.* **2020**, *7*, MMT39. [CrossRef] [PubMed]
5. Jacquelot, N.; Pitt, J.M.; Enot, D.P.; Roberti, M.P.; Duong, C.P.M.; Rusakiewicz, S.; Eggermont, A.M.; Zitvogel, L. Immune biomarkers for prognosis and prediction of responses to immune checkpoint blockade in cutaneous melanoma. *Oncoimmunology* **2017**, *6*, e1299303. [CrossRef]
6. Locati, M.; Curtale, G.; Mantovani, A. Diversity, Mechanisms, and Significance of Macrophage Plasticity. *Annu. Rev. Pathol.* **2020**, *15*, 123–147. [CrossRef] [PubMed]
7. Chevrier, S.; Levine, J.H.; Zanotelli, V.R.T.; Silina, K.; Schulz, D.; Bacac, M.; Ries, C.H.; Ailles, L.; Jewett, M.A.S.; Moch, H.; et al. An Immune Atlas of Clear Cell Renal Cell Carcinoma. *Cell* **2017**, *169*, 736–749.e718. [CrossRef]
8. Lavin, Y.; Kobayashi, S.; Leader, A.; Amir, E.D.; Elefant, N.; Bigenwald, C.; Remark, R.; Sweeney, R.; Becker, C.D.; Levine, J.H.; et al. Innate Immune Landscape in Early Lung Adenocarcinoma by Paired Single-Cell Analyses. *Cell* **2017**, *169*, 750–765.e717. [CrossRef]
9. Sica, A.; Mantovani, A. Macrophage plasticity and polarization: In vivo veritas. *J. Clin. Investig.* **2012**, *122*, 787–795. [CrossRef]
10. Yang, M.; McKay, D.; Pollard, J.W.; Lewis, C.E. Diverse Functions of Macrophages in Different Tumor Microenvironments. *Cancer Res.* **2018**, *78*, 5492–5503. [CrossRef]
11. Barros, M.H.; Hauck, F.; Dreyer, J.H.; Kempkes, B.; Niedobitek, G. Macrophage polarisation: An immunohistochemical approach for identifying M1 and M2 macrophages. *PLoS ONE* **2013**, *8*, e80908. [CrossRef]
12. Ladanyi, A. Prognostic and predictive significance of immune cells infiltrating cutaneous melanoma. *Pigment Cell Melanoma Res.* **2015**, *28*, 490–500. [CrossRef]
13. Jensen, T.O.; Schmidt, H.; Moller, H.J.; Hoyer, M.; Maniecki, M.B.; Sjoegren, P.; Christensen, I.J.; Steiniche, T. Macrophage markers in serum and tumor have prognostic impact in American Joint Committee on Cancer stage I/II melanoma. *J. Clin. Oncol.* **2009**, *27*, 3330–3337. [CrossRef]
14. Storr, S.J.; Safuan, S.; Mitra, A.; Elliott, F.; Walker, C.; Vasko, M.J.; Ho, B.; Cook, M.; Mohammed, R.A.; Patel, P.M.; et al. Objective assessment of blood and lymphatic vessel invasion and association with macrophage infiltration in cutaneous melanoma. *Mod. Pathol.* **2012**, *25*, 493–504. [CrossRef] [PubMed]
15. Tremble, L.F.; McCabe, M.; Walker, S.P.; McCarthy, S.; Tynan, R.F.; Beecher, S.; Werner, R.; Clover, A.J.P.; Power, X.D.G.; Forde, P.F.; et al. Differential association of CD68(+) and CD163(+) macrophages with macrophage enzymes, whole tumour gene expression and overall survival in advanced melanoma. *Br. J. Cancer* **2020**, *123*, 1553–1561. [CrossRef] [PubMed]
16. Hanahan, D.; Weinberg, R.A. Hallmarks of cancer: The next generation. *Cell* **2011**, *144*, 646–674. [CrossRef]
17. Samaniego, R.; Gutierrez-Gonzalez, A.; Gutierrez-Seijo, A.; Sanchez-Gregorio, S.; Garcia-Gimenez, J.; Mercader, E.; Marquez-Rodas, I.; Aviles, J.A.; Relloso, M.; Sanchez-Mateos, P. CCL20 Expression by Tumor-Associated Macrophages Predicts Progression of Human Primary Cutaneous Melanoma. *Cancer Immunol. Res.* **2018**, *6*, 267–275. [CrossRef]
18. Baade, P.; Meng, X.; Youlden, D.; Aitken, J.; Youl, P. Time trends and latitudinal differences in melanoma thickness distribution in Australia, 1990–2006. *Int. J. Cancer* **2012**, *130*, 170–178. [CrossRef] [PubMed]
19. Samaniego, R.; Estecha, A.; Relloso, M.; Longo, N.; Escat, J.L.; Longo-Imedio, I.; Aviles, J.A.; del Pozo, M.A.; Puig-Kroger, A.; Sanchez-Mateos, P. Mesenchymal contribution to recruitment, infiltration, and positioning of leukocytes in human melanoma tissues. *J. Investig. Dermatol.* **2013**, *133*, 2255–2264. [CrossRef]
20. Gutierrez-Gonzalez, A.; Martinez-Moreno, M.; Samaniego, R.; Arellano-Sanchez, N.; Salinas-Munoz, L.; Relloso, M.; Valeri, A.; Martinez-Lopez, J.; Corbi, A.L.; Hidalgo, A.; et al. Evaluation of the potential therapeutic benefits of macrophage reprogramming in multiple myeloma. *Blood* **2016**, *128*, 2241–2252. [CrossRef]
21. Estecha, A.; Sanchez-Martin, L.; Puig-Kroger, A.; Bartolome, R.A.; Teixido, J.; Samaniego, R.; Sanchez-Mateos, P. Moesin orchestrates cortical polarity of melanoma tumour cells to initiate 3D invasion. *J. Cell Sci.* **2009**, *122*, 3492–3501. [CrossRef]
22. Langmead, B.; Salzberg, S.L. Fast gapped-read alignment with Bowtie 2. *Nat. Methods* **2012**, *9*, 357–359. [CrossRef]
23. Li, B.; Dewey, C.N. RSEM: Accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinform.* **2011**, *12*, 323. [CrossRef] [PubMed]
24. Subramanian, A.; Tamayo, P.; Mootha, V.K.; Mukherjee, S.; Ebert, B.L.; Gillette, M.A.; Paulovich, A.; Pomeroy, S.L.; Golub, T.R.; Lander, E.S.; et al. Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 15545–15550. [CrossRef] [PubMed]
25. Liberzon, A.; Birger, C.; Thorvaldsdottir, H.; Ghandi, M.; Mesirov, J.P.; Tamayo, P. The Molecular Signatures Database (MSigDB) hallmark gene set collection. *Cell Syst.* **2015**, *1*, 417–425. [CrossRef]

26. Edgar, R.; Domrachev, M.; Lash, A.E. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res.* **2002**, *30*, 207–210. [CrossRef]
27. Donadon, M.; Torzilli, G.; Cortese, N.; Soldani, C.; Di Tommaso, L.; Franceschini, B.; Carriero, R.; Barbagallo, M.; Rigamonti, A.; Anselmo, A.; et al. Macrophage morphology correlates with single-cell diversity and prognosis in colorectal liver metastasis. *J. Exp. Med.* **2020**, *217*. [CrossRef] [PubMed]
28. Gartrell, R.D.; Marks, D.K.; Hart, T.D.; Li, G.; Davari, D.R.; Wu, A.; Blake, Z.; Lu, Y.; Askin, K.N.; Monod, A.; et al. Quantitative Analysis of Immune Infiltrates in Primary Melanoma. *Cancer Immunol. Res.* **2018**, *6*, 481–493. [CrossRef]
29. Lumeng, C.N.; Bodzin, J.L.; Saltiel, A.R. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J. Clin. Investig.* **2007**, *117*, 175–184. [CrossRef] [PubMed]
30. McGovern, N.; Schlitzer, A.; Gunawan, M.; Jardine, L.; Shin, A.; Poyner, E.; Green, K.; Dickinson, R.; Wang, X.N.; Low, D.; et al. Human dermal CD14(+) cells are a transient population of monocyte-derived macrophages. *Immunity* **2014**, *41*, 465–477. [CrossRef]
31. Wunderlich, C.M.; Ackermann, P.J.; Ostermann, A.L.; Adams-Quack, P.; Vogt, M.C.; Tran, M.L.; Nikolajev, A.; Waisman, A.; Garbers, C.; Theurich, S.; et al. Obesity exacerbates colitis-associated cancer via IL-6-regulated macrophage polarisation and CCL-20/CCR-6-mediated lymphocyte recruitment. *Nat. Commun.* **2018**, *9*, 1646. [CrossRef] [PubMed]
32. Smith, M.P.; Sanchez-Laorden, B.; O'Brien, K.; Brunton, H.; Ferguson, J.; Young, H.; Dhomen, N.; Flaherty, K.T.; Frederick, D.T.; Cooper, Z.A.; et al. The immune microenvironment confers resistance to MAPK pathway inhibitors through macrophage-derived TNFalpha. *Cancer Discov.* **2014**, *4*, 1214–1229. [CrossRef] [PubMed]
33. Porta, C.; Rimoldi, M.; Raes, G.; Brys, L.; Ghezzi, P.; Di Liberto, D.; Dieli, F.; Ghisletti, S.; Natoli, G.; De Baetselier, P.; et al. Tolerance and M2 (alternative) macrophage polarization are related processes orchestrated by p50 nuclear factor kappaB. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 14978–14983. [CrossRef]
34. Hagemann, T.; Lawrence, T.; McNeish, I.; Charles, K.A.; Kulbe, H.; Thompson, R.G.; Robinson, S.C.; Balkwill, F.R. "Re-educating" tumor-associated macrophages by targeting NF-kappaB. *J. Exp. Med.* **2008**, *205*, 1261–1268. [CrossRef]
35. Lowe, J.M.; Menendez, D.; Bushel, P.R.; Shatz, M.; Kirk, E.L.; Troester, M.A.; Garantziotis, S.; Fessler, M.B.; Resnick, M.A. p53 and NF-kappaB coregulate proinflammatory gene responses in human macrophages. *Cancer Res.* **2014**, *74*, 2182–2192. [CrossRef] [PubMed]

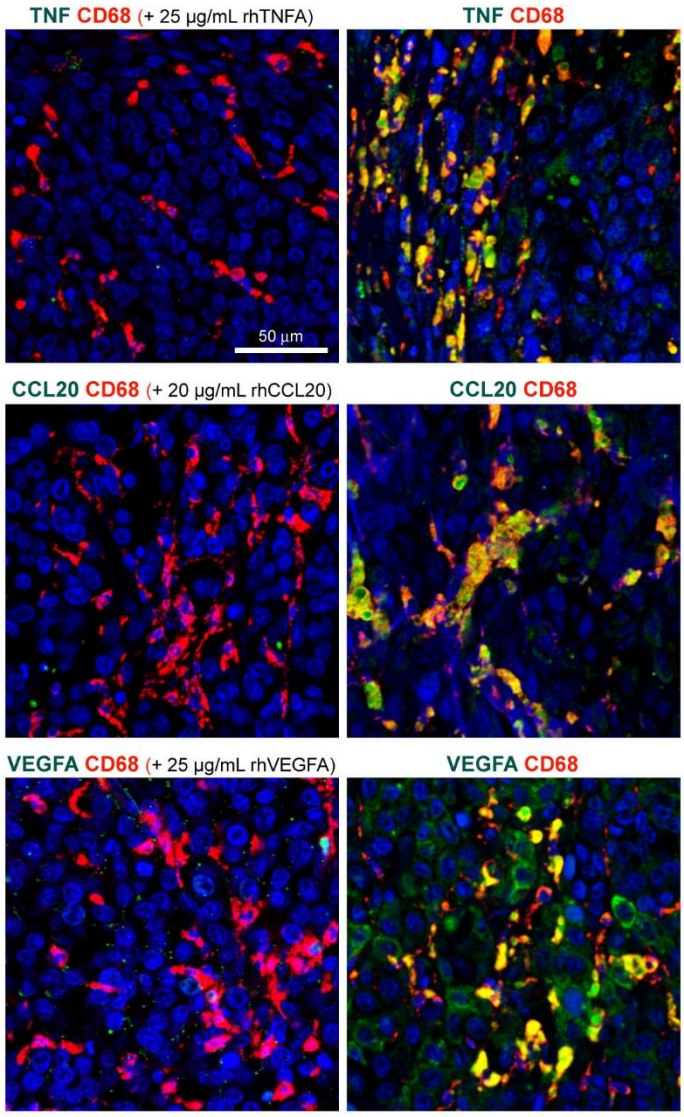


Figure S1: Validation of staining specificity

Table S1: Frozen and paraffin-embedded primary melanoma collections, clinicopathological features.

PARAFFIN	**FROZEN**
Gender	**Gender**
Male (n=45)	Male (n=27)
Female (n=38)	Female (n=13)
Age (years)	**Age** (years)
≤65 (n=36)	≤65 (n=20)
>65 (n=47)	>65 (n=20)
Location	**Location**
Head/limbs (n=58)	Head/limbs (n=15)
Trunk (n=25)	Trunk (n=25)
Subtype	**Subtype**
Nodular (n=30)	Nodular (n=14)
Others (n=53)	Others (n=26)
Ulceration	**Ulceration**
No (n=42)	No (n=26)
Yes (n=36)	Yes (n=14)
Breslow	**Breslow**
≤4mm (n=38)	≤2mm (n=19)
>4mm (n=45)	>2mm (n=21)
Stage	**Stage**
II (n=61)	I-II (n=33)
III (n=16)	III (n=7)
IV (n=6)	
Metastasis (10 years)	**Metastasis** (5 years)
No (n=32)	No (n=24)
Yes (n=51)	Yes (n=16)
Survival (10 years)	**Survival** (5 years)
Yes (n=50)	Yes (n=32)
No (n=33)	No (n=8)

Table S2: Association of evaluated prognostic markers with clinicopathological features (Mann-Whitney).

		TAM CCL20	P	TAM TNF	P	TAM VEGFA	P	TC VEGFA	P
Gender	Male (n=45)	106 ± 5	0.011	112 ± 6	0.125	102 ± 5	0.089	74 ± 5	0.165
	Female (n=38)	85 ± 5		99 ± 4		93 ± 6		64 ± 6	
Age (years)	≤65 (n=36)	89 ± 6	0.215	102 ± 5	0.780	88 ± 6	0.043	61 ± 5	0.204
	>65 (n=47)	97 ± 4		104 ± 4		104 ± 5		73 ± 5	
Location	Head/limbs (n=58)	93 ± 4	0.866	102 ± 4	0.615	99 ± 5	0.849	69 ± 5	0.577
	Trunk (n=25)	95 ± 6		106 ± 6		94 ± 5		65 ± 6	
Subtype	Nodular (n=30)	87 ± 5	0.158	109 ± 5	0.261	95 ± 5	0.922	68 ± 7	0.782
	Others (n=53)	97 ± 5		100 ± 4		98 ± 5		68 ± 4	
Ulceration	No (n=42)	91 ± 5	0.75	104 ± 5	0.827	102 ± 6	0.39	64 ± 5	0.218
	Yes (n=36)	95 ± 5		105 ± 5		94 ± 5		74 ± 6	
Breslow	≤4mm (n=38)	95 ± 5	0.806	105 ± 5	0.582	100 ± 5	0.562	69 ± 5	0.786
	>4mm (n=45)	92 ± 5		101 ± 5		94 ± 5		66 ± 5	
Stage	II (n=63)	87 ± 4	0.003	99 ± 4	0.025	86 ± 5	0.002	67 ± 5	0.565
	III-IV (n=20)	113 ± 7		117 ± 6		112 ± 7		72 ± 6	
Metastasis (10 years)	No (n=32)	78 ± 6	<0.001	85 ± 4	<0.001	78 ± 6	<0.001	56 ± 5	0.014
	Yes (n=51)	105 ± 5		118 ± 5		110 ± 5		77 ± 5	
Survival (10 years)	Yes (n=50)	89 ± 5	0.113	94 ± 4	<0.001	88 ± 5	0.007	64 ± 5	0.318
	No (n=33)	101 ± 5		121 ± 5		111 ± 5		75 ± 6	

La activina A sustenta el fenotipo metastásico de los macrófagos asociados a tumores y es un marcador de pronóstico en el melanoma cutáneo humano

Las células tumorales atraen e interactúan dinámicamente con monocitos/macrófagos para subvertir su diferenciación en macrófagos asociados a tumor (TAM), que promueven principalmente la supresión inmunitaria y la progresión neoplásica, pero las vías y las señales microambientales que gobiernan su desviación pro-tumoral no se conocen por completo. Para identificar las vías moleculares responsables de la diferenciación de TAM, analizamos los biomarcadores secretados durante las interacciones entre melanoma y macrófagos utilizando microarrays de Quantibody y secuenciación de RNA de macrófagos. Descubrimos que la activina A, un miembro de la familia TGF, juega un papel fundamental en la comunicación cruzada entre las células de melanoma y los monocitos/macrófagos, lo que resulta en la regulación al alza de distintos genes sustentadores de tumores y el logro de funciones pro-invasivas e inmunosupresoras de TAM. El bloqueo de la activina reduce la regulación positiva de parte de estos genes e impide la adquisición de funciones pro-tumorales, lo que facilita el rechazo del melanoma humano por linfocitos humanos transferidos en un modelo de ratón con xenoinjerto. Sorprendentemente, la detección de dos colecciones independientes de melanoma primario cutáneo mostró que la activina A está enriquecida en TAM y células de melanoma de pacientes con peor pronóstico y constituye un marcador de pronóstico nuevo e independiente. Por lo tanto, identificamos a la activina A como un intermediario clave en las funciones pro-tumorales e inmunosupresoras de los TAM, con un potencial significativo como biomarcador de enfermedades y como diana inmunoterapéutica.

Gutiérrez-Seijo A, García-Martínez E, Barrio-Alonso C, Parra-Blanco V, Avilés-Izquierdo JA, Sánchez-Mateos P, Samaniego R. Activin A Sustains the Metastatic Phenotype of Tumor-Associated Macrophages and Is a Prognostic Marker in Human Cutaneous Melanoma. J Invest Dermatol. 2022 Mar;142(3 Pt A):653-661.e2. doi: 10.1016/j.jid.2021.07.179. Epub 2021 Sep 6. PMID: 34499901.

ORIGINAL ARTICLE

Activin A Sustains the Metastatic Phenotype of Tumor-Associated Macrophages and Is a Prognostic Marker in Human Cutaneous Melanoma

Alba Gutiérrez-Seijo[1,2], Elena García-Martínez[1,2], Celia Barrio-Alonso[1,2], Verónica Parra-Blanco[3], José Antonio Avilés-Izquierdo[4], Paloma Sánchez-Mateos[2,5,6] and Rafael Samaniego[1,6]

Tumor cells attract and dynamically interact with monocytes/macrophages to subvert their differentiation into tumor-associated macrophages (TAMs), which mainly promote immune suppression and neoplastic progression, but the pathways and microenvironmental cues governing their protumoral deviation are not completely understood. To identify the molecular pathways responsible for TAM differentiation, we screened the biomarkers secreted during melanoma–macrophage interactions using Quantibody microarrays and RNA sequencing of macrophages. We found that activin A, a member of the transforming GF family, plays an instrumental role in the cross-talk between melanoma cells and monocytes/macrophages, which results in the upregulation of distinct tumor-sustaining genes and the achievement of proinvasive and immunosuppressive functions of TAMs. Blockade of activin reduces the upregulation of part of these genes and prevents the acquisition of protumoral functions, facilitating human melanoma rejection by transferred human lymphocytes in a xenograft mouse model. Remarkably, screening of two independent cutaneous primary melanoma collections showed that activin A is enriched in TAMs and melanoma cells from patients with worse outcomes and constitutes a new and independent prognostic marker. Thus, we identify activin A as a key intermediary in the protumoral and immunosuppressive functions of TAMs, with significant potential as a disease biomarker as well as an immunotherapeutic target.

Journal of Investigative Dermatology (2021) ■, ■–■; doi:10.1016/j.jid.2021.07.179

INTRODUCTION

Melanoma is the most lethal of cutaneous malignancies, and pathological evaluation of primary skin melanomas, which assesses Breslow level of invasion, ulceration, and mitotic index, does not completely define the prognosis of patients (Gershenwald and Scolyer, 2018; Schadendorf et al., 2018). Accurate prognosis is important in patients with cutaneous melanoma to guide adjuvant immunotherapy, and new biomarkers are urgently needed (Rizk et al., 2020; Schuchter, 2017). Information derived from analysis of the melanoma microenvironment may serve as a biomarker to identify patients with a high risk of metastatic spreading.

Tumor-associated macrophages (TAMs) are abundant cells in the tumor microenvironment that establish complex paracrine interactions with cancer cells and other leukocytes, displaying great plasticity and multiple protumoral or antitumoral functions (Locati et al., 2020). TAMs may originate from the proliferation of resident macrophages, but active recruitment of monocytes (Mos) and subverted local differentiation to TAMs are recognized as the predominant source of TAMs (Franklin and Li, 2016). An important chemokine axis driving Mos recruitment to most tumors is CCR2/CCL2 (Qian et al., 2011), which is operative in melanoma, where CCL2 is expressed by stromal cells (Samaniego et al., 2013). We have also described a CCR6/CCL20 paracrine axis between TAMs expressing CCL20 and CCR6^{+} melanoma cells (Samaniego et al., 2018). CCL20 expression by TAMs predicts the progression of human primary cutaneous melanoma, which also express TNF and VEGFA cytokines, suggesting a differential TAM activation state.

In this study, to characterize the oncogenic activation of human macrophages, we performed a biomarker screening, identifying activin as a secreted factor governing TAM deviation. Activins are members of the TGF family that share signaling pathways with TGFβ, explaining some of the overlapping functions of these cytokines (Loomans and Andl, 2014). Members of TGFβ superfamily act on various components of the tumor stroma and have been previously reported to mediate tumor–stroma interactions and antitumor immunity (Batlle and Massagué, 2019). Thus, depending on the context, TGFβ signaling facilitates or inhibits tumor

[1]*Unidad de Microscopía Confocal, Instituto de Investigación Sanitaria Gregorio Marañón, Madrid, Spain;* [2]*Laboratorio de Inmuno-oncología, Instituto de Investigación Sanitaria Gregorio Marañón, Madrid, Spain;* [3]*Servicio de Anatomía Patológica, Hospital General Universitario Gregorio Marañón, Madrid, Spain;* [4]*Servicio de Dermatología, Hospital General Universitario Gregorio Marañón, Madrid, Spain; and* [5]*Departamento de Inmunología, Facultad de Medicina, Universidad Complutense de Madrid, Madrid, Spain*

[6]*These authors contributed equally to this work.*

Correspondence: Rafael Samaniego, Unidad de Microscopía Confocal, Instituto de Investigación Sanitaria Gregorio Marañón, c/ Maiquez, 9, 28009 Madrid, Spain. E-mail: confocal@hggm.es

Abbreviations: ACT, adoptive cell transfer; CM, conditioned media; Mo, monocyte; TAM, tumor-associated macrophage; TC, tumor cell

Received 3 April 2021; revised 10 June 2021; accepted 26 July 2021; accepted manuscript published online XXX; corrected proof published online XXX

progression (Bellomo et al., 2016). Activins are homodimers or heterodimers composed of two β inhibin chains, being the most abundant variant activin A (homodimer βAβA, referred to as activin in the remaining part of this paper).

RESULTS

Oncogenic activation of macrophages: Screening of secreted factors

TAMs are known to release products that directly support tumor cell (TC) growth or modify the local microenvironment (Yang et al., 2018). To search for secreted factors during melanoma/macrophage coculture, we mixed BLM melanoma cells with macrophage colony–stimulating factor–differentiated macrophages and quantified soluble proteins in the supernatants using Quantibody microarrays (RayBiotech, Peachtree Corners, GA), including cytokines, chemokines, GFs, soluble receptors, and others (Supplementary Table S1). To address the cell source of secreted factors, melanoma-conditioned macrophages were separated and analyzed by RNA sequencing, compared with resting macrophages. Among the group of soluble factors induced during melanoma/macrophage coculture, we found high secretion of the TGF family member activin, whose corresponding gene *INHBA* (coding for βA chain of activin) was upregulated in melanoma-conditioned macrophages (Figure 1a and Supplementary Figure S1a). Other members of this family, such as TGFα, TGFβ1, TGFβ2, TGFβ3, were not induced in melanoma/macrophage supernatants, and therefore, we focused our interest on activin that is a key regulator of inflammatory and immune responses (Morianos et al., 2019). Next, we studied peripheral blood Mos because these cells are directly recruited to tumors to become TAMs (Yang et al., 2018). We measured activin by ELISA in supernatants of Mos cocultured with three melanoma cell lines (BLM, A375, and Skmel-103), showing enhanced secretion by Mo/BLM and Mo/A375 (Supplementary Figure S1b). After 3 or 24 hours of coculture, melanoma-educated Mos were processed for qPCR analysis, finding *INHBA* upregulation by coculture with BLM and A375, whereas no change was detected in *TGFB1* (Supplementary Figure S1c). Melanoma-educated Mos showed an upregulation of the protumoral signatures *CCL20*, *TNF*, and *VEGFA* mRNA (Samaniego et al., 2018) as

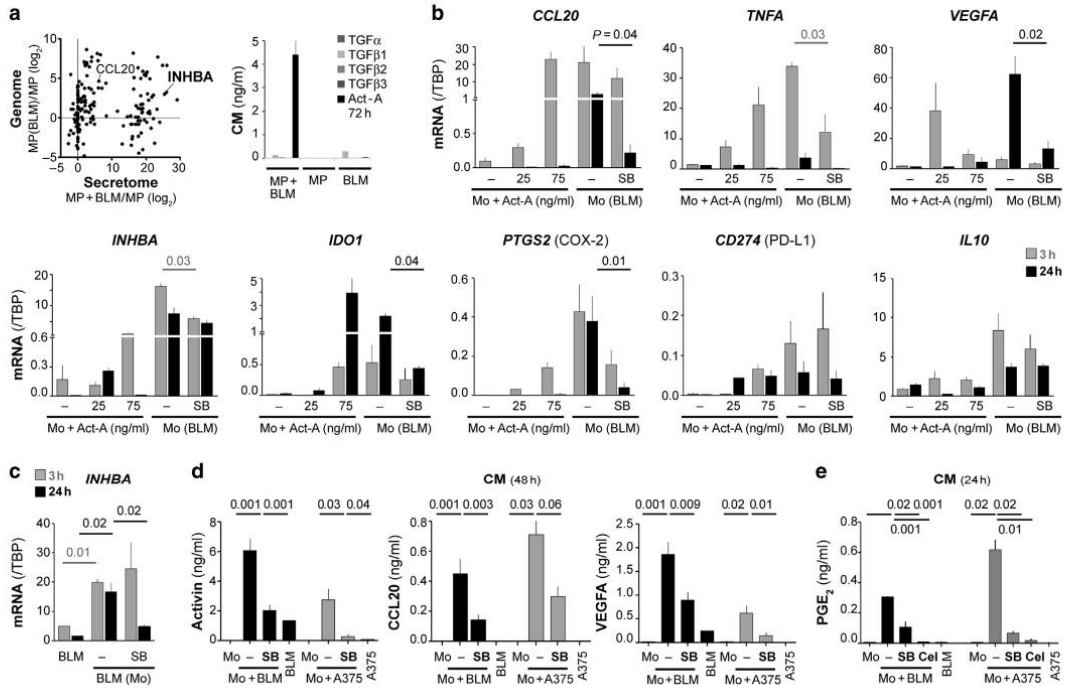


Figure 1. Activin contribution to in vitro reprogramming of MPs/Mo. (**a**) Secretomic versus genomic analysis: logarithmic fold change variations of 198 secreted proteins and their corresponding genes quantified in 72 h (co)cultured supernatants of MCSF-differentiated MPs + BLM cells versus of MPs alone (n = 4 donors) or at isolated BLM-conditioned MP versus unconditioned MP (co)cultured for 24 h (n = 3), respectively. TGF family proteins secretion is shown as means ± SD. (**b**) Mos incubated with human activin or mixed with BLM cells in the presence/absence of SB-431542. Genes expression is shown as means ± SD mRNA values relative to those of *TBP* (n = 3–6). (**c**) *INHBA* mRNA expression assessed at BLM cells conditioned by Mos in the presence/absence of SB-431542 for 3 (gray) or 24 (black) h, as indicated (n = 3–4). (**d**) activin, CCL20, and VEGFA secretion for 48 h assessed at Mos/melanoma cells CM, as indicated (n = 3–5). (**e**) PGE_2 secretion for 24 h in the presence/absence of SB-431542 or COX-2 inhibitor Celecoxib (for comparison), as indicated (n = 3–5). SW033291 inhibitor was present in all cases to avoid PGE_2 degradation. Paired *t*-test statistical analysis values are shown. Act-A, activin A; Cel, celecoxib; CM, conditioned media; h, hour; MCSF, macrophage colony–stimulating factor; Mo, monocyte; MP, macrophage; PGE_2, prostaglandin E_2; SB, SB-431542.

well as other factors involved in immunosuppressive properties of TAMs: cytokines such as *IL10* (coding for IL-10), enzymes such as *IDO1* (coding for indoleamine 2,3-dioxygenase 1) and *PTGS2* (coding for cyclooxygenase-2 or COX-2), or ligands such as *CD274* (coding for PD-L1) (Supplementary Figure S1c). Altogether, these results indicated that melanoma conditioning of Mos/macrophages induced the upregulation of the inflammatory cytokine activin, together with *CCL20, TNF*, and *VEGF* oncogenic signature and other immunosuppressive factors.

Activin mediates melanoma conditioning of Mos

Because it was reported that activin promotes carcinogenesis by attraction and reprogramming of macrophages in a mouse model of nonmelanoma skin cancer (Antsiferova et al., 2017), we checked the role of activin in driving melanoma conditioning of human Mos/macrophages. We first checked that melanoma conditioning of Mos induced SMAD2 nuclear translocation, indicating signaling through TGFβ family cytokines (Supplementary Figure S1d). Similar to melanoma conditioning of Mos, a dose-dependent treatment with activin induced mRNA upregulation of the protumoral signatures *CCL20, TNF*, and *VEGFA* as well as *IDO1* and *PTGS2* immunosuppressive factors (Figure 1b). In addition, activin treatment induced its own gene (*INHBA)* expression, generating a feedforward-activating mechanism of Mos/macrophages. We also used the inhibitor of ALK5, ALK4, and ALK7, SB-431542, which inhibited ligand-dependent SMAD2 phosphorylation (Supplementary Figure S1d), to explore the contribution of TGFβ/activin signaling to melanoma conditioning of Mos. Upregulation of *CCL20, TNF, VEGFA, INHBA, PTGS2*, and *IDO1* was abolished by 60−90%, indicating that TGFβ/activin signaling was an intermediary in their induction, whereas no inhibition was observed for *IL10* and *CD274* at the mRNA level, indicating TGFβ/activin independence (Figure 1b). On the other side, *INHBA* mRNA expression was also induced in BLM melanoma cells by coculture with Mos, an effect that could be reversed by SB-431542, indicating cross-activation and feedforward activin induction by melanoma cells (Figure 1c). In accordance with mRNA expression data, both the inhibitor SB-431542 and neutralizing anti-activin antibody reduced the secretion of activin, CCL20, and VEGFA proteins by over 60% in melanoma/Mo supernatants (Figure 1d and Supplementary Figure S1e). Furthermore, prostaglandin E_2, the product of the COX-2, was reduced in melanoma/Mo supernatants by blocking with SB-431542 at a level comparable with that of the specific COX-2 inhibitor celecoxib (Figure 1e). All these data indicate that activin is a key intermediate factor in Mo–melanoma crosstalk because it is actively secreted during initial interactions and because its signaling controls the secretion of other protumoral and immunosuppressive factors.

Activin drives the acquisition of immunosuppressive and protumoral functions of melanoma-educated Mos

We next analyzed the ability of melanoma/Mos conditioned media (CM) to suppress T-cell proliferation. For suppression assays, we stimulated polyclonal proliferation of resting lymphocytes with anti-CD3/CD28–coated beads and assessed T-cell proliferation by carboxyfluorescein diacetate succinimidyl ester dye dilution. Whereas the CM from Mos or melanoma cell lines grown alone had no negative impact on T-lymphocyte expansion, T-cell proliferation was inhibited by CM from Mo/BLM and Mo/A375 but not by Mo/Skmel-103 cocultures (Figure 2a), which is in line with the failure of Skmel-103 cells in inducing activin and immunosuppressive factors in coculture with Mos (Supplementary Figure S1b and c). T-cell suppression was predominantly mediated by the presence of secreted factors in the CM because adding activin directly did not affect T-cell proliferation (Figure 2a). To determine the involvement of TGFβ/Activin signaling, we used SB-431542 during melanoma conditioning of Mos; then, Mos were separated from melanoma cells and cocultured with proliferating T cells for 72 hours. We observed that BLM-conditioned Mos inhibited T-cell proliferation in a dose-dependent fashion, whereas activin signaling inhibition during melanoma conditioning of Mos abolished their suppressive effect on T-cell proliferation (Figure 2b), supporting a role for activin in the acquisition of T-cell suppressive function by melanoma-educated Mos/macrophages.

Increased motility and invasion are hallmarks of tumor metastasis (Welch and Hurst, 2019). To evaluate whether melanoma/Mo CM could promote this capability, we performed melanoma three-dimensional invasion assays through collagen matrices. Melanoma spheroids were grown for 7−10 days, embedded into collagen type-I networks, and allowed to invade in the presence of different CM. CM from Mos cocultured with BLM or A375 cells significantly induced three-dimensional invasion of melanoma spheroids (Figure 2c). Remarkably, the blockade of activin with an antibody or its signaling with SB-431542 during melanoma/Mo coculture abolished the proinvasive properties of CM (Figure 2c). Dose-dependent three-dimensional invasion of melanoma spheroids was induced with prostaglandin E_2, TNF, and CCL20, which are factors induced during melanoma–Mo interactions. By contrast, adding activin directly did not significantly affect melanoma invasion, indicating that the effects of activin are indirect (Figure 2c). Our data indicate that after its secretion, activin controls the induction of immunosuppressive and proinvasive functions of melanoma-conditioned Mos.

Activin is enriched in TAMs and TCs of patients with melanoma with worse evolution

Our screening identified activin as a key cytokine induced during melanoma–myeloid cross-talk; furthermore, we showed that its secretion mediates the immunosuppressive and proinvasive functions of melanoma-educated Mos/macrophages in vitro. To explore whether TAMs and/or cancer cells constitute an important source of activin in vivo, fractions including each cell type were isolated from advanced primary melanomas (n = 3 cutaneous tumors of stage IV patients), finding mRNA expression of *INHBA*, whereas Mos from the blood of these advanced-stage patients showed no *INHBA* expression (Figure 2d), indicating that activin secretion may be a local event that takes place in the tumor microenvironment but not a general systemic conditioning of myeloid cells. Next, we explored the clinical significance of activin expression in the microenvironment of human skin melanoma. We analyzed the tissue content of activin in two independent cohorts of patients with primary cutaneous

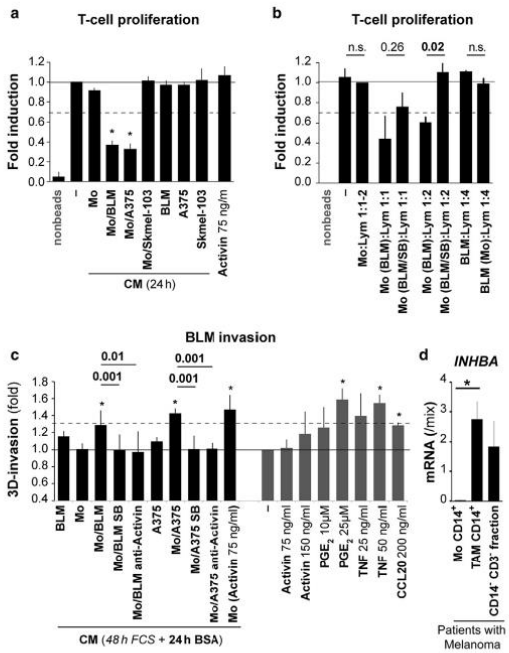


Figure 2. Activin contribution to the achievement of protumor functionality. (**a**) T-cell proliferation induced by anti-CD3/CD28 microbeads for 72 h in the presence of CM from Mos mixed or not with distinct melanoma cell lines for 24 h (n = 3–6 donors) or medium supplemented with human activin. Data relative to unconditioned medium (fold = 1) are presented. (**b**) Induced proliferation of lymphocytes mixed with Mos alone or Mos conditioned by BLM cells in the presence/absence of SB-431542 or with BLM cells alone or conditioned by Mos, as indicated (n = 3–5). Data relative to Mos-alone condition (fold = 1) are presented. Mo- or melanoma-to-Lym ratios are indicated. Representative CFSE fading experiments as assessed by flow cytometry. (**c**) BLM collagen 3D invasion performed in the presence of distinct serum-free Mo/melanoma CM (n = 3–5) or in media supplemented with distinct compounds, as indicated. 72 h invasions were performed in duplicate (coculture) or triplicate (compounds). (**d**) *INHBA* expression assessed at $CD14^+$ blood Mos and TAMs and at the remaining $CD14^-$ $CD3^-$ tumor fraction freshly isolated from patients with stage IV melanoma (n = 3). Means ± SD mRNA values relative to those of *TBP/GAPDH* average are shown. Paired *t*-test statistical analyses values are shown; $^*P < 0.05$ (relative to respective controls). 3D, three-dimensional; CFSE, carboxyfluorescein diacetate succinimidyl ester; CM, conditioned media; h, hour; Lym, lymphocyte; Mo, monocyte; n.s., not significant; PGE_2, prostaglandin E_2; TAM, tumor-associated macrophage.

melanoma: a first cohort of 40 patients and a validation cohort of 83 patients. Primary tissue samples were classified as nonmetastatic or metastatic regarding subsequent development of metastasis during patient follow-up of 5 and 10 years in each cohort, respectively. Primary melanomas were cryopreserved in the first cohort and were formalin fixed and paraffin embedded in the validation one, and therefore, different conditions were set up to perform multicolor confocal microscopy and quantification at the single-cell level of activin content. The best gating of macrophages for image processing and semiautomatic quantification was obtained with CD163 in cryopreserved samples, whereas CD68 was used for TAM segmentation in paraffin samples (Figure 3a and b and c–f, respectively). CD163 and CD68 are coexpressed in over 95% TAMs, together with CD115 and CD14, indicating that they are suitable pan-macrophage markers (Supplementary Figure S1f and g) (Samaniego et al., 2018). Quantification of the mean fluorescence intensity of activin βA subunit was developed in both cohorts along macrophages and TCs and was compared with clinicopathological factors; higher activin expression, in macrophages and melanoma cells, correlated with metastatic progression during follow up in both series of patients ($P < 0.001$) (Supplementary Table S2). Next, to perform survival analysis using the Kaplan–Meier method, patients were grouped as high or low activin content in either TAMs or TCs using the median mean fluorescence intensity value as the cutoff point, showing significant differences between the two groups in both patient cohorts; high activin in TAMs or TCs correlated with shorter disease-free survival (Figure 3b and f; log-rank test, both $P < 0.001$). In addition, TGFβ1, IDO1, and COX-2 were evaluated in the formalin-fixed cohort, finding differences in TAM COX-2 content between metastatic and nonmetastatic samples (Figure 3c). Single-cell measurements of TGFβ1 and activin are also shown for individual macrophages in nonmetastatic and metastatic cases, finding a subpopulation of TAMs highly expressing activin in metastatic samples (Figure 3e). Finally, to determine whether activin content in TAMs or TCs was an independent prognostic factor, we performed a multivariate regression analysis, including sex, age, Breslow, or stage parameters (Table 1 and Supplementary Table S3, for each patient cohort). These analyses showed that activin content in TAMs and/or TCs were independent prognostic factors of disease-free survival (both cohorts) and overall survival (in the larger validation cohort). These findings highlighted the clinical value of activin content in melanoma microenvironment, which may be a useful biomarker for the prognosis of patients with primary cutaneous melanoma.

Activin is a potential target for immunotherapy in a T-cell–based allogeneic rejection model

To explore the potential therapeutic value of targeting activin as a critical intermediary in the cross-talk between melanoma and myeloid cells, we set up a T-cell–based immunotherapy model of tumor rejection, which is driven by adoptive cell transfer (ACT) of human lymphocytes in immunodeficient NOD/Scid/$Il2r\gamma^{-/-}$ mice bearing human melanoma tumors (Figure 4a), as previously reported (Hennequart et al., 2017; Sanmamed et al., 2015). First, we evaluated the impact of inoculating subcutaneously BLM human melanoma cells mixed with human Mos (mixed tumors), finding that in the absence of an adaptive immune system, the initial presence of human Mos did not modify tumor growth. Next, mice engrafted with human melanoma cells were subsequently treated with ACT 4 days after tumor inoculation, finding significant smaller tumors, indicating that xenografted human melanoma is controlled by transferred allogeneic human lymphocytes ($P = 0.003$). By contrast, the therapeutic effect of ACT was lost in the mixed melanoma/Mo tumors, which doubled the size of melanoma-alone grafts treated with ACT ($P = 0.012$), indicating that intratumoral Mos confer

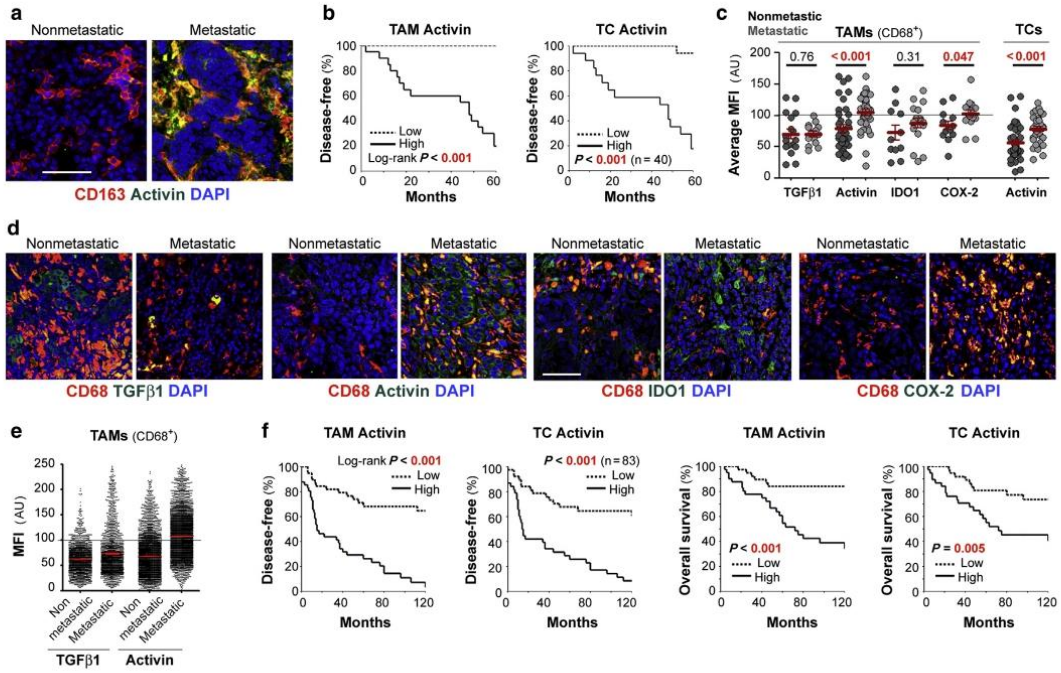


Figure 3. Confocal analysis of in vivo protein expression and correlation with patient evolution. (**a**) CD163 (red) and activin (green) staining at cryopreserved sections of nonmetastatic and metastatic primary melanomas. (**b**) 5-year disease-free Kaplan–Meier curves discriminating high or low activin expression by $CD163^+$ TAMs or TCs (frozen collection, n = 40 patients). Log-rank *P*-values are shown. (**c**) Plots showing the average MFIs (AU) of different proteins; each spot represents a paraffin-embedded biopsy, as indicated. Statistical differences are shown (Mann–Whitney). (**d**) CD68 (red) and quantified proteins (green) staining at sections of paraffin primary melanomas. (**e**) Plots showing single-TAM expression of TGFβ1 and activin in primary melanomas, as indicated. (**f**) A 10-year disease-free and overall survival Kaplan–Meier curves discriminating high and low activin expression at $CD68^+$ TAMs and TCs from the paraffin-embedded cohort (n = 83 patients). Log-rank *P*-values are shown. For all images, bar = 50 μm. AU, arbitrary unit; MFI, mean fluorescence intensity; TAM, tumor-associated macrophage; TC, tumor cell.

resistance to ACT immunotherapy (Figure 4b). Thus, we used a specific anti-activin blocking antibody injected every 3 days to study the role of this cytokine in three different situations (Figure 4c). In the absence of ACT therapy, anti-activin antibody treatment resulted in slower progression of mixed melanoma/Mo tumors than of isotype-control antibody-treated mice, although it was not statistically significant ($P = 0.061$). Interestingly, these differences were significant when mice treated with anti-activin antibody also received ACT immunotherapy ($P = 0.004$). Finally, antitumor effects of ACT therapy were similar in melanoma-alone tumors, independently of anti-activin or control antibody treatment ($P = 0.14$), indicating that host cells contributed minimally to activin effects in this model (Figure 4c). These results were further supported in a parallel set of experiments using A375 melanoma cells and SB-431542 inhibitor, which resulted in slower progression of A375 melanoma/Mo mixed tumors in NOD/Scid/*Il2rg*$^{-/-}$ mice plus ACT immunotherapy ($P = 0.007$) but not in mice engrafted with A375 melanoma cells alone (Figure 4d), which reinforces that blocking activin signaling relieves ACT function, depending on the previous presence of intratumoral human Mos (mixed tumors). These results support that in tumors rich in myeloid cells, a suppressive microenvironment orchestrated by activin confers resistance to T-cell–based immunotherapy, and therefore, blocking activin signaling in combined treatments may help to overcome resistance to immunotherapy.

DISCUSSION

Melanoma is one of the most immunogenic tumors and was the first human cancer in which immune checkpoint inhibitors showed clinical efficacy (Schadendorf et al., 2018). However, melanoma is also characterized by a highly immunosuppressive microenvironment, which is involved in the primary or acquired resistance to these agents. Among other regulatory leukocytes, TAMs exert potent immunesuppressive functions in the tumor microenvironment, expressing inhibitory checkpoint receptors and producing factors that modulate the antitumoral response of other components of the immune response (Gordon et al., 2017). In this work, we screened the biomarkers of melanoma–macrophage cross-talk and identified activin, which on its secretion drives the expression of many protumoral factors and orchestrates immunosuppressive and invasive functions of melanoma-educated Mos/macrophages. Our data in

Table 1. Univariate and Multivariate Cox-Regression Analyses for Disease-Free and Overall Survival (Validation Paraffin Collection)

Univariate	Disease-Free Survival			Overall Survival		
Sex (F vs. M)	1.602	0.91–2.81	0.100	1.168	0.55–2.47	0.685
Age (y)	1.003	0.99–1.02	0.704	1.007	0.98–1.03	0.574
Location (H/L vs. T)	1.452	0.74–2.85	0.278	0.895	0.40–1.99	0.785
Subtype (nod vs. others)	0.831	0.46–1.52	0.547	1.305	0.60–2.82	0.498
Ulceration (yes vs. no)	1.354	0.76–2.41	0.301	2.582	1.15–5.82	0.022
Breslow (mm)	1.130	1.07–1.20	<0.001	1.124	1.04–1.21	0.002
Stage (II vs. III–IV)	1.865	1.19–2.92	0.006	1.793	0.74–4.33	0.194
TAM activin (MFI, each 10 AU)	1.174	1.08–1.28	<0.001	1.132	1.03–1.25	0.012
TC activin (MFI, each 10 AU)	1.280	1.15–1.43	<0.001	1.243	1.08–1.43	0.002
Multivariate	**HR**	**95% CI**	***P***	**HR**	**95% CI**	***P***
Sex (F vs. M)	1.153	0.65–2.05	0.627	0.622	0.28–1.40	0.253
Age (y)	1.007	0.99–1.03	0.472	1.012	0.98–1.04	0.387
Breslow (mm)	1.162	1.09–1.23	<0.001	1.169	1.07–1.28	0.001
Stage (II vs. III–IV)	0.954	0.52–1.77	0.882	0.850	0.28–2.56	0.773
TAM activin (MFI, each 10 AU)	1.207	1.11–1.32	<0.001	1.199	1.08–1.33	0.001
Sex (F vs. M)	1.43	0.77–2.64	0.256	0.812	0.35–1.86	0.623
Age (y)	1.015	0.99–1.04	0.210	1.013	0.99–1.04	0.368
Breslow (mm)	1.131	1.04–1.23	0.005	1.178	1.08–1.29	<0.001
Stage (II vs. III–IV)	1.175	0.61–2.26	0.628	0.987	0.36–2.68	0.979
TC activin (MFI, each 10 AU)	1.324	1.18–1.49	<0.001	1.350	1.17–1.56	<0.001

Abbreviations: AU, arbitrary unit; CI, confidence interval; F, female; H/L, head/limb; HR, hazard ratio; M, male; MFI, mean fluorescence intensity; nod, nodular; T, trunk; TAM, tumor-associated macrophage; TC, tumor cell.

human melanoma tissues indicate that activin is expressed by TAMs and TCs in certain primary skin melanomas and is associated with a worse patient prognosis. Importantly, we quantitatively analyzed activin tissue content by multicolor confocal microscopy in two cohorts of patients with primary cutaneous melanoma, finding that high activin expression by TAMs and TCs in primary tumors is an independent prognostic factor for metastatic progression of patients with cutaneous melanoma. As a new disease biomarker, activin expression by primary tumors may be useful to guide adjuvant immunotherapy in cutaneous melanoma because in the adjuvant setting, only patients with a higher risk of developing metastasis will benefit, whereas all treated patients, with a lower or higher risk of metastasis, may suffer adverse events (Rizk et al., 2020; Schuchter, 2017).

Various members of the TGFβ family acting on TCs and their microenvironment play complex context-dependent signaling in cancer. Thus, the relative contribution of autocrine versus paracrine activin signaling, mediating protective or tumorigenic effects, appears to be cancer type specific. For example, in pancreatic cancer, activin receptor ALK4 (also known as ACVR1B) mediates tumor-suppressive functions and is frequently deleted in clinical samples (Togashi et al., 2014). By contrast, circulating activin is frequently upregulated in lung or colorectal adenocarcinomas, being a biomarker to predict poor prognosis (Hoda et al., 2016; Wu et al., 2015). In skin cancers, activin is highly expressed in human basal and squamous cell carcinomas and in melanoma cell lines and melanoma tumors (Antsiferova et al., 2011; Heinz et al., 2015; Hoek et al.,

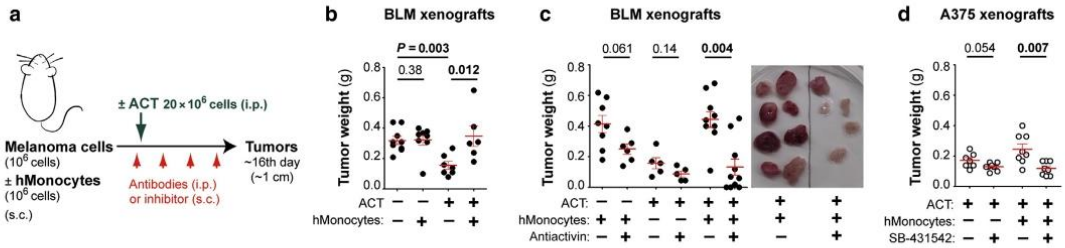


Figure 4. Activin targeting in combination with T-cell–based immunotherapy to overcome resistance in a myeloid-rich melanoma model. (**a**) Scheme of ACT mouse model. (**b**) BLM cells mixed or not with human monocytes xenografts developed in NSG mice transferred or not with human PBMCs, as indicated. Tumor weight values are shown (n = 6–8 mice). (**c**) Xenograft weights, as in **b**, from mice i.p. injected with blocking anti-activin (+) or isotype (−) antibodies, as indicated (n = 5–9). (**d**) A375 cells mixed or not with human monocytes xenografts grown in NSG mice reconstituted with human lymphocytes and s.c. injected or not with SB-431542, as indicated (n = 6–8). Mann–Whitney statistical analyses are shown. ACT, adoptive cell transfer; hMonocytes, human monocytes; i.p., intraperitoneally; NSG, NOD/Scid/*Il2rg*$^{-/-}$; s.c., subcutaneously.

2006). Activin overexpression in keratinocytes was tumorigenic in nonmelanoma skin cancer model through its paracrine action on stromal cells, particularly by attraction of blood Mos and reprogramming of a protumorigenic macrophage phenotype (Antsiferova et al., 2017, 2011). In melanoma, the gain of transgenic activin expression in the B16 mouse model was tumorigenic, but no changes in total myeloid populations were observed (Donovan et al., 2017). A paracrine effect of activin on NK cells driving their differentiation into a less cytotoxic cell type was recently reported in the B16 model, showing that blockade of activin improves NK cell function and antitumor immunity (Rautela et al., 2019). Activin signaling is also required for the suppressive function of regulatory T cells, and blockade of the activin in the B16 melanoma model resulted in dysfunctional regulatory T cells unable to suppress antitumor immunity (Ni et al., 2018). In this study, we show that activin is actively secreted during melanoma–macrophage crosstalk, and it is a key factor regulating protumoral and immunosuppressive functions of melanoma-educated Mos. Our results in human melanoma, together with recent reports in the B16 mouse melanoma model, indicate that multiple immune cell types may be targeted by activin to establish local suppressive environments, including Mo/macrophage, NK, and regulatory T cells.

In this study, we have identified the cell source of activin in human melanoma tissues, which is expressed by both TCs and TAMs, and have assessed its clinical utility as a prognostic factor in cutaneous melanoma. These findings, together with our previous work in melanoma (Samaniego et al., 2018), indicate that activin and CCL20 expression by macrophages is indicative of a particular secretory phenotype, which is highlighted in prometastatic TAMs. Our results support an amplifying mechanism in which activin, induced during melanoma–Mo crosstalk, initiates a feedforward loop that controls its own expression as well as a vast array of factors, such as immunosuppressive COX-2/prostaglandin E_2 (Mao et al., 2013) and IDO1 (Hennequart et al., 2017), prosurvival TNF (Smith et al., 2014), proinvasive CCL20 (Samaniego et al., 2018), or proangiogenic VEGFA (Apte et al., 2019). In addition to reinforcing protumoral TAM differentiation in an autocrine manner, activin, owing to its high diffusibility (McDowell et al., 1997), may promote paracrine local effects in newly recruited Mos as well as in other immune target cells. Therefore, activin should be considered as a potential target for improving immunotherapies in melanoma. To prove this hypothesis, we used a humanized T-cell–based therapy model of ACT to treat human melanoma xenografts in NOD/Scid/*Il2rg*$^{-/-}$ mice. We first showed that infusion of allogeneic lymphocytes controlled the growth of two human melanoma xenografts. However, the therapeutic antitumoral effect of ACT was lost in mixed tumors rich in Mos/macrophages. These data suggested that Mos/macrophages in mixed tumors suppressed the allogeneic rejection and prevented the immunotherapy response to ACT. Interestingly, the ACT antitumoral response was recovered by activin blockade in mixed xenografts. By contrast, in tumors originated from melanoma cells alone, activin blockade did not alter tumor progression. Thus, activin targeting appears to be complementary to immunotherapy in a tumor context rich in myeloid cells to relieve immune-suppressive microenvironments created by melanoma/Mo interactions. Our results support that activin is an instrumental cytokine orchestrating the local protumoral and immunosuppressive microenvironment of human melanoma and uncover the potential targeting of activin signaling to improve immunotherapy.

MATERIAL AND METHODS

Mo isolation and cell culture

PBMCs were isolated from patients or buffy coats from healthy donors over a ficoll (Lymphocytes Isolation Solution, Rafer, Zaragoza, Spain) gradient. Mos were purified by magnetic cell sorting using anti-CD14–tagged microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and were cultured at 0.5×10^6/ml. All cell types were cultured in RMPI-1640 medium (Gibco, Waltham, MA) supplemented with 10% fetal calf serum (Sigma-Aldrich, St. Louis, MO), unless otherwise specified. BLM, A375, and Skmel-103 human melanoma cell lines (Estecha et al., 2009; Samaniego et al., 2018, 2013) were not reauthenticated during the last year but were checked for melanoma markers (HMB45 and NG2).

Human melanoma samples

Tissues from biopsied primary large lesions were homogenized and digested into single-cell suspensions (Tumor Dissociation Kit, Miltenyi Biotec). TAMs and tumor-infiltrating lymphocytes were purified by magnetic cell sorting using CD14 and CD3 microbeads, respectively. The remaining negatively selected cells were considered as TCs (Samaniego et al., 2018, 2013).

In vitro measurements

Melanoma cells were cocultured with Mos at a 1:2 ratio (melanoma-to-Mo ratio). Supernatants were collected for CCL20, VEGFA, activin, TNF, and prostaglandin E_2 secretion assessment (R&D Systems, Minneapolis, MN), and cells were processed for qPCR analysis, separating conditioned Mos from TCs with CD14 microbeads. Oligonucleotides (Supplementary Table S4) were designed according to the Roche software for qPCR (Roche Diagnostics, Basel, Switzerland). RNA was extracted (NucleoSpin RNA-purification kit; Macherey-Nagel, Dueren, Germany), and retrotranscribed cDNA was quantified using the Universal Human Probe Roche library (Roche Diagnostics). Assays were made in triplicate and normalized to TBP and/or *GAPDH* expression (ΔΔCT method). Western blot, immunofluorescence, and flow cytometry analyses were performed as previously described (Estecha et al., 2009; Gutiérrez-González et al., 2016; Samaniego et al., 2018, 2013). A comparative screening by multiplex ELISA arrays (Quantibody) and stranded transcriptome resequencing (RNA sequencing, BGI, Tai Po Industrial Estate, Hong Kong) was performed, coculturing BLM cells and M-CSF–differentiated macrophages for 72 hours, prepared as previously described (Gutiérrez-González et al., 2016; Samaniego et al., 2018). When specified, recombinant human activin A (Immunotools), blocking anti-activin A antibody (1 μg/ml; polyclonal; R&D Systems), goat IgG isotype–control antibody (1 μg/ml; R&D Systems), ALK4/5/7 inhibitor (SB-431542, 25 μM; 30 minutes pretreatment, Sigma-Aldrich), COX-2 inhibitor (Celecoxib, 10 μM; Sigma-Aldrich), or 15-hydroxy prostaglandin dehydrogenase inhibitor (SW033291, 1 μM; MedChemExpress, Monmouth Junction, NJ), were added every 24 hours.

In vitro suppression assay

Lymphocytes negatively isolated from PBMCs with CD14 microbeads were labeled with 1 μM carboxyfluorescein diacetate succinimidyl

ester and cultured (10^5 cells per 200 µl RPMI medium supplemented with 10% fetal calf serum and 50 U/ml IL-2) with Dynabeads Human T-Activator CD3/CD28 (Gibco) at a cell-to-bead ratio of 1:1. Lymphocytes were cultured in CM or with conditioned cells for 72 hours, and cellular proliferation (carboxyfluorescein diacetate succinimidyl ester fading) was measured by flow cytometry.

Three-dimensional invasion assay

BLM spheroids were produced by seeding 10^4 cells per well in a 96-well U-bottom plate coated with 100 µl of 1% agar for 7–10 days, embedded into 2 mg/ml collagen type-I matrices, and allowed to invade in the presence of serum-deprived CM. These media were prepared by coculturing BLM and Mos as previously described for 48 hours and then replacing the medium with RMPI medium supplemented with 1% BSA for 24 hours. For quantification, cells were fixed/permeabilized with 2% formaldehyde/0.25% Triton X-100 in PBS, stained with propidium iodide (Sigma-Aldrich), and imaged at the spheroid middle section with an inverted confocal microscope (HC-PL-APO ×10/NA 0.4 objective; SP2, Leica Microsystems, Wetzlar, Germany), considering five random migration lengths by spheroid with the LCS software (Leica Microsystems).

Melanoma xenograft models

NOD/Scid/*Il2rg*$^{-/-}$ mice (The Jackson Laboratory, Bar Harbor, ME) were maintained under specific pathogen–free conditions. At age of 4–6 weeks (~23 g), male mice were subcutaneously inoculated at both mouse flanks with 10^6 melanoma cells mixed or not with 10^6 human Mos suspended in PBS. Four days afterward, mice were intraperitoneally injected (ACT) or not with 20×10^6 human PBMCs (BLM model) or lymphocytes negatively selected for CD14 (A375 model). When tumors reached approximately 1 cm width, mice were killed by cervical dislocation, and tumors were resected and weighed. For in vivo activin blockade assays, BLM cells and Mos were inoculated in 100 µl PBS containing 500 ng/ml blocking anti-human/mouse inhibin βA subunit or goat IgG isotype-control antibodies (R&D Systems) and then intraperitoneally injected with 10 µg antibodies every 3 days. In a second model, A375 cells and Mos were inoculated in PBS containing or not 25 µM SB-431542, and then mice were subcutaneously injected with 100 µl PBS containing none or 500 µM SB-431542 every 2 days. Procedures were approved by the Instituto de Investigación Sanitaria Gregorio Marañón (Madrid, Spain) animal care/use and Comunidad de Madrid committees (PROEX 084/18).

Histological analysis by immunofluorescence

Two cutaneous primary melanomas collections with known clinicopathological features (Supplementary Table S2) were used to evaluate the tissue expression of different proteins: a previously used frozen collection of 40 patients (Samaniego et al., 2018) and a second cohort of 83 patient biopsies preserved in formaldehyde and embedded in paraffin along the years 1998–2015 (median follow-up of 68 months, range = 4–260 months; Breslow > 2 mm). Approaches included written informed patient consent and were approved by the Hospital General Universitario Gregorio Marañón (Madrid, Spain) ethical committee. Paraffin-embedded samples were deparaffinized, rehydrated, and unmasked by steaming for 6 minutes in indicated pH buffers (Supplementary Table S4) (Dako, Glostrup, Denmark). Slides were blocked with 5 µg/ml human immunoglobulins solved in blocking serum-free medium (Dako) for 30 minutes and then sequentially incubated with 5–10 µg/ml primary antibodies in PBS containing 10% blocking medium overnight at 4 °C and proper fluorescent secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA) for 1 hour at room temperature. Intermediate washes were performed in agitation and by immersion in PBS containing 0.05% Tween-20. Samples were imaged with a confocal microscope using the glycerol-immersion ACS APO ×20/NA 0.60 objective (SPE, Leica). At least three blindly chosen fields were quantified by sample.

Single-cell quantification was performed as previously described (Gutiérrez-González et al., 2016; Samaniego et al., 2018, 2013). For proper TAM segmentation, whole-cell area staining is necessary to define TAMs contour, which was achieved by CD163 and CD68 antibodies at frozen and paraffin-embedded sections, respectively. Mean fluorescence intensity of stained proteins was obtained at manually depicted TC nests or at each segmented TAM using the analyzed particle plugging of Fiji software.

Statistical analyses

GraphPad (GraphPad Software, San Diego, CA) or Statistical Package for the Social Sciences (IBM, Armonk, NY) programs were used for statistical analysis; tests are specified through the text.

Data availability statement

Datasets can be found at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE171277, hosted at Gene Expression Omnibus (GSE171277).

ORCIDs

Alba Gutiérrez-Seijo: http://orcid.org/0000-0001-6282-3932
Elena García-Martínez: http://orcid.org/0000-0001-8211-4287
Celia Barrio-Alonso: http://orcid.org/0000-0002-2789-1363
Verónica Parra-Blanco: http://orcid.org/0000-0002-7916-5478
José Antonio Avilés-Izquierdo: http://orcid.org/0000-0001-9451-4392
Paloma Sánchez-Mateos: http://orcid.org/0000-0001-6589-4445
Rafael Samaniego: http://orcid.org/0000-0002-3081-7332

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank Julia Villarejo, José María Bellón, Laura Díaz, and Fernando Asensio as well as the Instituto de Investigación Sanitaria Gregorio Marañón facilities (Madrid, Spain) for expert technical assistance and/or discussions. This work was partially supported by the Ministry of Science and Innovation ISCIII-FIS grant PI17/01324 (PSM and RS) cofinanced by ERDF/FEDER Funds from the European Commission—A way of making Europe—and by Cancer Research UK, FCAECC (GCB15152947MELE), and AIRC under the Accelerator Award Program. AGS and CBA were financed by the Comunidad de Madrid YEI program.

AUTHOR CONTRIBUTIONS

Conceptualization: AGS, PSM, RS; Data Curation: AGS, VPB, JAAI; Formal Analysis: AGS, PSM, RS; Funding Acquisition: PSM, RS; Investigation: AGS, EGM, CBA, RS; Methodology: RS; Project Administration: AGS, EGM, CBA, RS; Resources: VPB, JAAI, PSM; Supervision: PSM, RS; Validation: AGS, EGM, CBA, RS; Writing - Original Draft Preparation: PSM, RS

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2021.07.179.

REFERENCES

Antsiferova M, Huber M, Meyer M, Piwko-Czuchra A, Ramadan T, MacLeod AS, et al. Activin enhances skin tumourigenesis and malignant progression by inducing a pro-tumourigenic immune cell response. Nat Commun 2011;2:576.

Antsiferova M, Piwko-Czuchra A, Cangkrama M, Wietecha M, Sahin D, Birkner K, et al. Activin promotes skin carcinogenesis by attraction and reprogramming of macrophages. EMBO Mol Med 2017;9:27–45.

Apte RS, Chen DS, Ferrara N. VEGF in signaling and disease: beyond discovery and development. Cell 2019;176:1248–64.

Batlle E, Massagué J. Transforming growth factor-β signaling in immunity and cancer. Immunity 2019;50:924–40.

Bellomo C, Caja L, Moustakas A. Transforming growth factor β as regulator of cancer stemness and metastasis. Br J Cancer 2016;115:761–9.

Donovan P, Dubey OA, Kallioinen S, Rogers KW, Muehlethaler K, Müller P, et al. Paracrine activin-A signaling promotes melanoma growth and metastasis through immune evasion. J Invest Dermatol 2017;137:2578–87.

Estecha A, Sánchez-Martín L, Puig-Kröger A, Bartolomé RA, Teixidó J, Samaniego R, et al. Moesin orchestrates cortical polarity of melanoma tumour cells to initiate 3D invasion. J Cell Sci 2009;122:3492–501.

Franklin RA, Li MO. Ontogeny of tumor-associated macrophages and its implication in cancer regulation. Trends Cancer 2016;2:20–34.

Gershenwald JE, Scolyer RA. Melanoma staging: American Joint Committee on Cancer (AJCC) 8th edition and beyond [published correction appears in Ann Surg Oncol 2018;25(Suppl. 3):993–4]. Ann Surg Oncol 2018;25:2105–10.

Gordon SR, Maute RL, Dulken BW, Hutter G, George BM, McCracken MN, et al. PD-1 expression by tumour-associated macrophages inhibits phagocytosis and tumour immunity. Nature 2017;545:495–9.

Gutiérrez-González A, Martínez-Moreno M, Samaniego R, Arellano-Sánchez N, Salinas-Muñoz L, Relloso M, et al. Evaluation of the potential therapeutic benefits of macrophage reprogramming in multiple myeloma. Blood 2016;128:2241–52.

Heinz M, Niederleithner HL, Puujalka E, Soler-Cardona A, Grusch M, Pehamberger H, et al. Activin A is anti-lymphangiogenic in a melanoma mouse model. J Invest Dermatol 2015;135:212–21.

Hennequart M, Pilotte L, Cane S, Hoffmann D, Stroobant V, Plaen E, et al. Constitutive IDO1 expression in human tumors is driven by cyclooxygenase-2 and mediates intrinsic immune resistance. Cancer Immunol Res 2017;5:695–709.

Hoda MA, Rozsas A, Lang E, Klikovits T, Lohinai Z, Torok S, et al. High circulating activin A level is associated with tumor progression and predicts poor prognosis in lung adenocarcinoma. Oncotarget 2016;7:13388–99.

Hoek KS, Schlegel NC, Brafford P, Sucker A, Ugurel S, Kumar R, et al. Metastatic potential of melanomas defined by specific gene expression profiles with no BRAF signature. Pigment Cell Res 2006;19:290–302.

Locati M, Curtale G, Mantovani A. Diversity, mechanisms, and significance of macrophage plasticity. Annu Rev Pathol 2020;15:123–47.

Loomans HA, Andl CD. Intertwining of activin A and TGFβ signaling: dual roles in cancer progression and cancer cell invasion. Cancers 2014;7:70–91.

Mao Y, Poschke I, Wennerberg E, Pico de Coaña Y, Egyhazi Brage S, Schultz I, et al. Melanoma-educated CD14+ cells acquire a myeloid-derived suppressor cell phenotype through COX-2-dependent mechanisms. Cancer Res 2013;73:3877–87.

McDowell N, Zorn AM, Crease DJ, Gurdon JB. Activin has direct long-range signalling activity and can form a concentration gradient by diffusion. Curr Biol 1997;7:671–81.

Morianos I, Papadopoulou G, Semitekolou M, Xanthou G. Activin-A in the regulation of immunity in health and disease. J Autoimmun 2019;104:102314.

Ni X, Tao J, Barbi J, Chen Q, Park BV, Li Z, et al. YAP is essential for Treg-mediated suppression of antitumor immunity. Cancer Discov 2018;8:1026–43.

Qian BZ, Li J, Zhang H, Kitamura T, Zhang J, Campion LR, et al. CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. Nature 2011;475:222–5.

Rautela J, Dagley LF, de Oliveira CC, Schuster IS, Hediyeh-Zadeh S, Delconte RB, et al. Therapeutic blockade of activin-A improves NK cell function and antitumor immunity. Sci Signal 2019;12:eaat7527.

Rizk EM, Seffens AM, Trager MH, Moore MR, Geskin LJ, Gartrell-Corrado RD, et al. Biomarkers predictive of survival and response to immune checkpoint inhibitors in melanoma. Am J Clin Dermatol 2020;21:1–11.

Samaniego R, Estecha A, Relloso M, Longo N, Escat JL, Longo-Imedio I, et al. Mesenchymal contribution to recruitment, infiltration, and positioning of leukocytes in human melanoma tissues. J Invest Dermatol 2013;133:2255–64.

Samaniego R, Gutiérrez-González A, Gutiérrez-Seijo A, Sánchez-Gregorio S, García-Giménez J, Mercader E, et al. CCL20 expression by tumor-associated macrophages predicts progression of human primary cutaneous melanoma. Cancer Immunol Res 2018;6:267–75.

Sanmamed MF, Rodriguez I, Schalper KA, Oñate C, Azpilikueta A, Rodriguez-Ruiz ME, et al. Nivolumab and urelumab enhance antitumor activity of human T lymphocytes engrafted in Rag2-/-IL2Rγnull immunodeficient mice. Cancer Res 2015;75:3466–78.

Schadendorf D, van Akkooi ACJ, Berking C, Griewank KG, Gutzmer R, Hauschild A, et al. Melanoma [published correction appears in Lancet 2019;393:746. Lancet 2018;392:971–84.

Schuchter LM. Adjuvant melanoma therapy - head-spinning progress. N Engl J Med 2017;377:1888–90.

Smith MP, Sanchez-Laorden B, O'Brien K, Brunton H, Ferguson J, Young H, et al. The immune microenvironment confers resistance to MAPK pathway inhibitors through macrophage-derived TNFα. Cancer Discov 2014;4:1214–29.

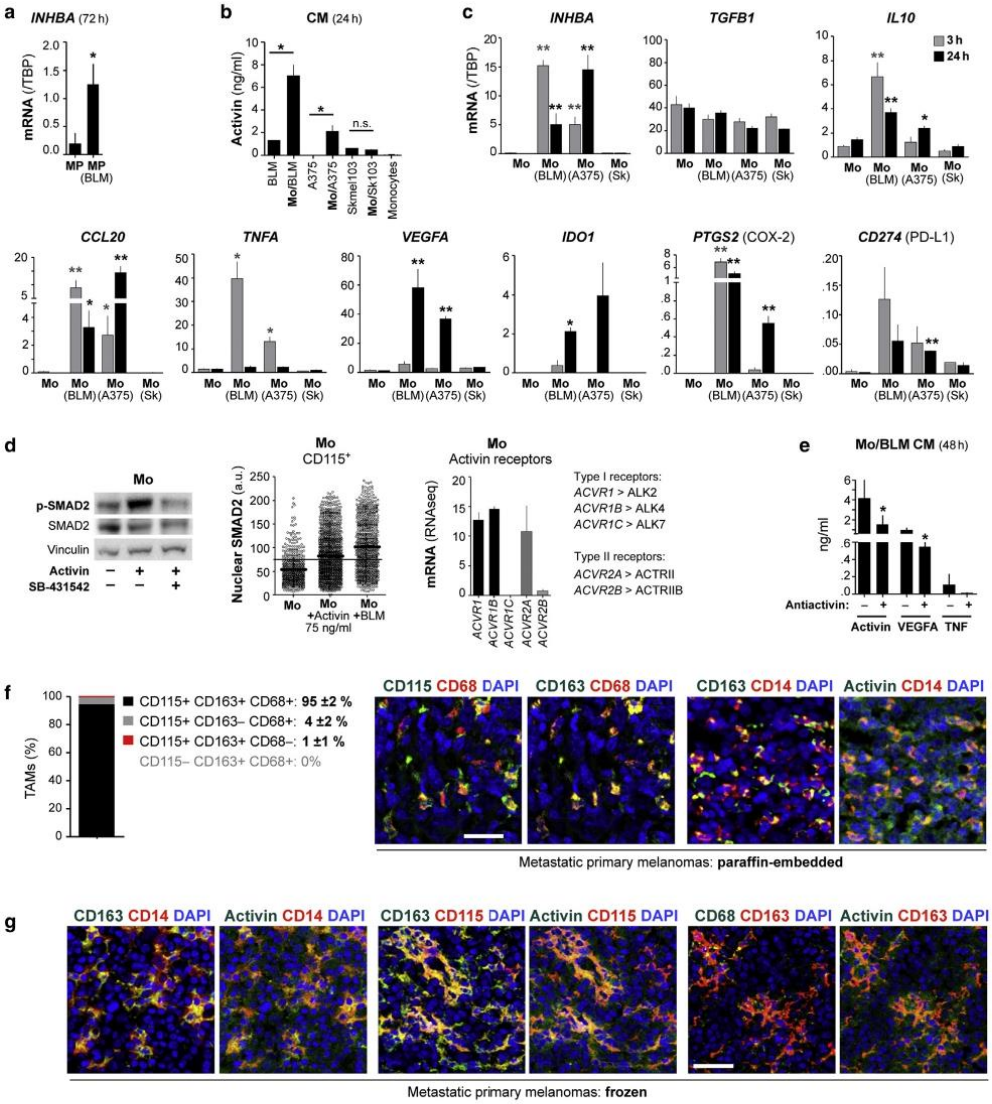
Togashi Y, Sakamoto H, Hayashi H, Terashima M, de Velasco MA, Fujita Y, et al. Homozygous deletion of the activin A receptor, type IB gene is associated with an aggressive cancer phenotype in pancreatic cancer. Mol Cancer 2014;13:126.

Welch DR, Hurst DR. Defining the hallmarks of metastasis. Cancer Res 2019;79:3011–27.

Wu S, Qi Y, Niu LM, Xie DX, Cui XL, Liu ZH. Activin A as a novel biomarker for colorectal adenocarcinoma in humans. Eur Rev Med Pharmacol Sci 2015;19:4371–8.

Yang M, McKay D, Pollard JW, Lewis CE. Diverse functions of macrophages in different tumor microenvironments. Cancer Res 2018;78:5492–503.

SUPPLEMENTARY MATERIALS



Supplementary Figure S1. Reprogramming of Mos by melanoma cells in vitro and in vivo. (**a**) MCSF-differentiated MP separated with CD14 beads after 72 h of coculture with BLM cells and analyzed for *INHBA* expression by qPCR. Means ± SD mRNA values relative to those of *TBP* are shown (n = 9 donors). (**b**) Activin (inhibin βA) secretion for 24 h, as indicated (n = 3–5). (**c**) Mos alone or mixed with BLM, A375, or Skmel-103 melanoma cells for 3 (gray) and 24 (black) h, separated with CD14 beads and analyzed by qPCR. Means ± SD mRNA values relative to those of *TBP* are shown (n = 3–9). (**d**) Left: Mos incubated or not with 75 ng/ml human activin in the presence or absence of SB-431542 inhibitor for 45 minutes. Immunoblots show phosphorylated SMAD2 (Ser465/467) and SMAD2 or vinculin as loading controls (representative blot of three experiments). Center: SMAD2 nuclear translocation assessed by immunofluorescence within DAPI-stained nuclei of $CD115^+$ Mos treated with 75 ng/ml activin or coincubated with BLM cells for 3 h. Right: activin receptors expressed by $CD14^+$ isolated Mos as assessed by RNA-seq (n = 3). (**e**) Activin, VEGFA, and TNF secretion for 48 h assessed at Mos/melanoma cells CM incubated or not with 1 μg/ml neutralizing anti-activin antibody, as indicated (n = 3–4). Paired *t*-test statistical analyses values are shown (**a**–**c**, **e**): $*P < 0.05$ and $**P < 0.005$. (**f**) Characterization of primary melanoma TAMs in paraffin-embedded samples. Over 95% TAMs coexpress CD68, CD163, and CD115 MP pan-markers (n = 4 biopsies; 1,000 TAMs), as previously shown in the frozen melanoma collection (Samaniego et al., 2018). Activin-expressing $CD163^+$ TAMs also coexpress CD14 MP marker, as indicated. (**g**) Activin-expressing TAMs coexpress CD163, CD68, CD115, and CD14 MP pan-markers in the cryosections of metastatic primary melanomas. Bar = 50 μm. AU, arbitrary unit; CM, conditioned media; h, hour; MCSF, macrophage colony–stimulating factor; Mo, monocyte; MP, macrophage; n.s., not significant; p-SMAD, phosphorylated SMAD; RNA-seq, RNA sequencing; TAM, tumor-associated macrophage.

SUPPLEMENTARY REFERENCE

Samaniego R, Gutiérrez-González A, Gutiérrez-Seijo A, Sánchez-Gregorio S, García-Giménez J, Mercader E, et al. CCL20 expression by tumor-associated macrophages predicts progression of human primary cutaneous melanoma. Cancer Immunol Res 2018;6:267–75.

DISCUSIÓN

El melanoma cutáneo es la más letal de las neoplasias cutáneas (Curti and Faries, 2021). Antes del advenimiento de la inmunoterapia como tratamiento, los pacientes con melanoma metastásico tenían muy mal pronóstico, con una supervivencia de sólo 6 a 10 meses (Balch *et al.*, 2009). Los tratamientos con inhibidores de los puntos de control inmune (immune checkpoint (ICP) inhibitors), que se desarrollaron inicialmente en melanoma metastásico también tienen el potencial de ser eficaces en el contexto adyuvante (en ausencia de enfermedad clínicamente evaluable), porque son más eficaces con una menor carga de la enfermedad, cuando la inmunosupresión mediada por el tumor es mínima (Schuchter, 2017). Aunque los inhibidores de los ICP han demostrado su eficacia en el melanoma, también se asocian a efectos adversos inmuno-mediados (EAI), que incluyen colitis, neumonitis y miocarditis (Postow et al., 2018). En el contexto metastásico, existe una mayor tolerancia para los EAI; sin embargo, en el entorno adyuvante, se necesita una mayor seguridad y no se pueden realizar ensayos en poblaciones grandes que incluyan muchos pacientes de bajo riesgo (Schuchter, 2017). Por lo tanto, se necesitan con urgencia biomarcadores de pronóstico para identificar qué pacientes tienen mayor riesgo de recurrencia para la estratificación de los ensayos clínicos (Jacquelot et al., 2017).

Las interacciones entre las células cancerosas y otras células o factores del microambiente tumoral van a tener un efecto importante en la biología tumoral (Hanahan and Weinberg, 2011). El estudio en profundidad del microambiente tumoral nos puede aportar las claves necesarias para comprender estas complejas interacciones y, finalmente, contribuir al establecimiento de herramientas de uso clínico. Por ello nuestro principal objetivo es identificar marcadores de macrófagos con un perfil pro-tumoral que permitan establecer un factor pronóstico sobre la evolución de pacientes de melanoma primario en estados I-III.

El análisis de imagen confocal multicolor nos permite cuantificar la expresión de diversos marcadores a nivel de células individuales, con la ventaja de tener información de localización dentro del tejido tumoral (borde tumoral, perivascular, necrosis) (Gutierrez-Gonzalez et al., 2016; Puig-Kroger et al., 2009; Samaniego et al., 2013; Samaniego et al., 2018; Samaniego et al., 2014; Sanchez-Martin et al., 2011). La información de localización dentro del microambiente tumoral es muy importante y se pierde en las técnicas que requieren disgregación tisular como la citometría, tanto la tradicional con fluoróforos como la citometría de masas. Además, los melanomas primarios son tumores generalmente pequeños, que se incluyen en su totalidad en

parafina para hacer el diagnóstico y no queda tumor suficiente para ser disgregado. Para la microscopía confocal multicolor se usan cantidades muy pequeñas de tejido, lo que permite realizar el diagnóstico convencional de anatomía patológica y su posterior análisis con inmunofluorescencia multicolor (IF multiplex).

Los estudios de tejidos humanos suelen utilizar inmunohistoquímica (IHC) cromogénica para evaluar proteínas específicas o marcadores celulares in situ; sin embargo, esta metodología solo es adecuada para el marcado sencillo o doble y la intensidad de la expresión de los marcados es difícilmente cuantificable. La IHC fluorescente y la microscopía espectral tienen la ventaja de la detección de múltiples marcadores y además las imágenes se pueden cuantificar y la intensidad de la fluorescencia nos da un valor relativo de la expresión de cada marcador. Un análisis profundo del TME puede proporcionar pistas para comprender las complejas interacciones de las células cancerosas con otras células o factores y, en última instancia, será de utilidad clínica para el establecimiento de nuevas herramientas de pronóstico o dianas terapéuticas. Con este fin, hemos puesto a punto la inmunofluorescencia multicolor, unida a la microscopía confocal, para estudiar in situ diversos componentes inmunes y estromales, así como la expresión de quimioquinas y sus receptores en el TME del melanoma humano. Esta metodología no destructiva del tejido nos permite estudiar la distribución espacial de diversas células, la ubicación de proteínas específicas dentro de las células o áreas de tejido y la cuantificación de su nivel relativo de expresión. El marcaje múltiple es de particular relevancia cuando se evalúan células heterogéneas como macrófagos, que se sabe que comparten marcadores con otras células mieloides o que expresan moléculas en relación con su polarización funcional.

Previamente se han establecido en nuestro laboratorio las condiciones para el marcado múltiple y el análisis cuantitativo de imágenes de TAM, en tejidos criopreservados o parafinados, que son más convenientes para el uso clínico. En primer lugar, como marcadores panmacrófagos auténticos, hemos demostrado que la mayoría de las células con morfología de macrófagos coexpresan CD163, CD68 y CD115. CD163 no define ningún subgrupo separado de TAM, lo que concuerda con su coexpresión con los marcadores M1 y M2 en patologías predominantes Th1 y Th2 (Barros et al., 2013). En nuestra experiencia, la definición de las subpoblaciones de TAM se consigue mejor mediante la tinción triple con un marcador panmacrófago, para segmentar los macrófagos, y la evaluación cuantitativa del nivel de expresión de CD11c y CD209 (Samaniego *et*

al., 2018). Aunque CD11c es un marcador muy utilizado para marcar células dendríticas convencionales (las DC CD11c+ "bona fide" no expresan marcadores de macrófagos y son poco frecuentes en melanoma), CD11c también se expresa en una subpoblación que coexpresa marcadores de macrófagos (CD163+/CD68++), que pueden corresponder a macrófagos M1.

Por otro lado, en nuestro trabajo Samaniego et al., 2018 también describimos cómo las células del melanoma expresan de manera generalizada el receptor CCR6 y cómo su ligando CCL20 solamente es expresado por los TAM de tumores primarios de pacientes que posteriormente desarrollan metástasis (<5 años). En este trabajo se utilizó una colección de 40 tumores primarios criopreservados y se describió además la existencia de TAM con un estado especial de activación que, aparte de CCL20, se caracterizan por la expresión de proteínas pro-tumorales como TNF y VEGFA. Este perfil pro-tumoral se detalló también en el trabajo de Chittezhath et al., 2014, en el que observaron que los monocitos de pacientes con carcinoma renal expresaban, entre otros genes, CCL20, TNF y VEGFA.

Con el fin de continuar sobre esta línea y validar este grupo de proteínas como marcadores independientes con valor pronóstico, pusimos a punto un estudio mediante inmunofluorescencia multicolor en una cohorte de 83 melanomas primarios incluidos en parafina. Los tejidos parafinados son más convenientes para el uso clínico ya que es el método más común para su conservación en hospitales y da acceso a un mayor número de muestras. Acorde con los resultados preliminares obtenidos en Samaniego et al., 2018, se observó que la producción de las citoquinas CCL20/TNF/VEGFA por TAM correlacionaba con una mala supervivencia de los pacientes. Para evaluar la importancia clínica de estos marcadores realizamos un estudio mediante curvas Kaplan-Meier y un análisis de regresión multivariante. Nuestros resultados validan este perfil pro-tumoral como un marcador pronóstico independiente de la evolución de melanoma.

Además, con el objetivo de profundizar en el estudio de estas citoquinas, procedimos a purificar los TAM y las células tumorales de 4 tumores primarios de pacientes en estadio IV para cuantificar la expresión de mRNA. Nuestros resultados demuestran que los TAM expresan activamente los genes *CCL20*, *TNF* y *VEGFA* comparado con los monocitos de sangre periférica aislados de estos pacientes.

Por otro lado, también quisimos estudiar otros parámetros asociados a TAM como son su densidad, su tamaño y su localización en el tumor, pero ninguna de estas características se asoció con valor pronóstico. También exploramos la posibilidad de que existiera una correlación entre la ratio de TAM M1/M2 y la supervivencia de los pacientes. Para ello utilizamos los marcadores de polaridad anteriormente mencionados, CD11c y CD209, y el pan-marcador CD163. Para analizar los resultados se representó la expresión de CD11c frente a CD209 en un dot plot. Se observó que en muestras control de piel la población dominante estaba formada por macrófagos CD209+ CD11c-, que probablemente se traten de los macrófagos residentes de tejido (McGovern et al., 2014). En cambio, en las muestras de melanoma primario observamos que aparece una nueva subpoblación CD209- CD11c+, seguramente formada por macrófagos pro-inflamatorios, y una subpoblación intermedia caracterizada por expresar tanto CD11c como CD209. Sin embargo, no se observaron diferencias significativas entre primarios metastásicos y no metastásicos, aunque sí se observó cierta tendencia a una menor proporción de macrófagos M2 en los melanomas primarios metastásicos.

Usando macrófagos derivados de monocitos, ya sea con GM-CSF o con M-CSF, que se sabe que se polarizan hacia los fenotipos M1 o M2 respectivamente (Samaniego *et al.*, 2014), producimos macrófagos condicionados por melanoma in vitro que expresan CCL20 y TNF (M1) o VEGFA (M2). Este sistema de co-cultivo in vitro de macrófagos con células de melanoma, nos permitió explorar las vías reguladoras subyacentes al fenotipo secretor de citoquinas CCL20 / TNF / VEGFA de TAM, demostrando que p53 y NF-κB corregulan macrófagos condicionados por tumores. Se sabe que la activación de NF-κB impulsa la polarización M2 de los TAM (Porta et al., 2009) y su inhibición reeduca a los TAM hacia un fenotipo anti-tumoral (Hagemann et al., 2008). De acuerdo con nuestros resultados, se describió que la activación dual de p53 y NF-κB en macrófagos induce la expresión de IL6, TNF y CCL20 (Lowe et al., 2014). Estos resultados identifican una vía p53 / NF-kB única en TAM pro-tumoral que podría ser apta para reeducar a los TAM y bloquear sus funciones pro-tumorales.

La producción de citoquinas por los macrófagos es un atributo de la polarización de los macrófagos (Sica and Mantovani, 2012); los M1 secretan grandes cantidades de citoquinas proinflamatorias (como IL-12, IL-23 y TNF), mientras que los M2 se caracterizan por la expresión de IL-10 y factores pro-angiogénicos como IL-8, VEGFA y VEGFC. Estas citoquinas son capaces de

modificar el microambiente tumoral e incluso fomentar el crecimiento del tumor (Yang et al., 2018b). Por ejemplo, la polarización de TAM secretores de CCL20 se inducía vía IL-6 en un modelo de ratón de cáncer de colon en el cual el CCL20 promovía la progresión del cáncer mediante el reclutamiento de linfocitos CCR6+ (Wunderlich et al., 2018). Por otro lado, el TNF derivado de macrófagos se identificó como un factor crucial en la resistencia a tratamientos con inhibidores de la vía MAPK en un modelo de ratón con melanoma mutado BRAFV600E (Smith et al., 2014).

Con el objetivo de buscar más factores secretados durante el co-cultivo de melanoma-macrófagos, decidimos condicionar la línea celular de melanoma BLM con macrófagos (diferenciados con M-CSF) y cuantificar un amplio espectro de proteínas mediante un microarray de anticuerpos llamado Quantibody. Este ensayo es capaz de medir cuantitativamente la presencia de múltiples citoquinas, factores de crecimiento, proteasas, receptores solubles y otras proteínas más en el sobrenadante del co-cultivo. Para determinar la fuente de las proteínas secretadas realizamos en paralelo una secuenciación del RNA de los macrófagos condicionados por melanoma y lo comparamos a macrófagos en estado de reposo. De entre todos los factores solubles analizados, nos llamó la atención una proteína de la familia de TGFβ llamada activina A. Esta proteína se encontraba en el sobrenadante en concentraciones elevadas y estaba además altamente expresada en los macrófagos condicionados por melanoma con respecto al control.

Es de sobra conocido que TGF-β juega un papel relevante en distintos tipos de tumor, incluyendo el melanoma, facilitando o inhibiendo su progresión dependiendo del contexto (Bellomo et al., 2016; Perrot et al., 2013). TGF-β es un potente inhibidor de la proliferación de las células epiteliales, y, por lo tanto, se considera un potente supresor tumoral durante los estadios iniciales de la carcinogénesis debido a estos efectos inhibitorios en el ciclo celular (Elliott and Blobe, 2005; Wakefield and Roberts, 2002). Sin embargo, durante la progresión de la enfermedad las células tumorales escapan de estos efectos inhibitorios de TGF-β e incluso, en estadios más tardíos, TGF-β ejerce un efecto pro-tumoral. Muchos tumores como el melanoma secretan grandes cantidades de TGF-β, que influye directamente en el microambiente y promueve el crecimiento tumoral, así como la angiogénesis, la invasión y la diseminación (Leivonen and Kahari, 2007). También se ha visto que TGF-β favorece el escape de la respuesta inmune a través de varios mecanismos inmunosupresores (Wan and Flavell, 2007).

Muchos de los atributos asociados a TGF-β se han observado también para la activina, aunque su efecto pleiotrópico va a depender de las características del microambiente tumoral (Batlle and Massague, 2019; Chen and Ten Dijke, 2016). De hecho, se ha visto que la activina A puede tener tanto efectos pro-tumorales como anti-tumorales según el tipo de cáncer (Antsiferova and Werner, 2012). Por ejemplo, la exposición a activina induce una inhibición en la proliferación y promueve la apoptosis en hepatocitos normales (Schwall et al., 1993) y también en líneas celulares de carcinoma de hígado (Vejda et al., 2003; Zauberman et al., 1997). Del mismo modo, la activina limita el crecimiento en líneas celulares de tumores epiteliales como el cáncer de próstata (Dalkin *et al.*, 1996), el cáncer de mama (Razanajaona *et al.*, 2007) o el cáncer de colon (Jung et al., 2007), e incluso también en el linfoma B de células grandes (Jiang et al., 2018) y en células endoteliales (Kaneda et al., 2011; Panopoulou et al., 2005). Sin embargo, también se ha observado una estimulación del crecimiento y de la supervivencia por parte de la activina en fibroblastos de pulmón (Ohga et al., 1996) y en líneas celulares de cáncer de pulmón (Seder *et al.*, 2009b) y de mesotelioma (Hoda et al., 2012). Mientras que la respuesta a activina con relación al crecimiento celular es ambivalente, la literatura es más categórica con respecto a la migración celular. Un gran número de estudios han establecido que la activina estimula la migración en varios tipos celulares, incluyendo algunos en los que se había observado una inhibición de la proliferación como en el cáncer de colon (Jung *et al.*, 2007). También se han reportado casos en los que la invasión y la metástasis se veían aumentadas a causa de la activina como es el caso del cáncer de próstata (Nomura *et al.*, 2013), de mama (Kalli *et al.*, 2019), el carcinoma oral de células escamosas (Chang *et al.*, 2010) y de mesotelioma (Tamminen *et al.*, 2015).

En cuanto al papel de la activina en melanoma, estudios previos han hallado que tanto las líneas celulares de melanoma como los tumores expresan frecuentemente activina (Heinz *et al.*, 2015; Hoek *et al.*, 2006). Heinz y colaboradores llevaron a cabo un estudio de expresión transgénica de activina en A375 para un modelo de ratón de melanoma humano y observaron un descenso en la linfangiogénesis tumoral. Sin embargo, no hubo alteraciones en el crecimiento tumoral ni en la metástasis. En otro estudio investigaron el efecto de la activina en melanoma murino de forma paracrina y autocrina, y también en ratones inmunocompetentes e inmunodeficientes. Los resultados obtenidos en este estudio indicaban que la activina podía

tener un efecto tumorigénico cuando actúa de forma paracrina en ratones inmunocompetentes, sugiriendo que la activina actúa sobre la respuesta inmune anti-tumoral inhibiéndola (Donovan *et al.*, 2017). Por otro lado, en un modelo de B16 se observó que la activina es requerida para la función supresora de las células T reguladoras, ya que cuando bloqueaban la activina estas células eran incapaces de reprimir la respuesta anti-tumoral, promoviendo un descenso en el crecimiento tumoral (Ni *et al.*, 2018). Por último, recientemente investigadores han examinado los efectos en las células NK en un modelo de B16 y han visto que la activina actúa de forma paracrina conduciendo la diferenciación de células NK hacia un fenotipo menos citotóxico, y mediante su bloqueo son capaces de recuperar la función citotóxica y su respuesta anti-tumoral (Rautela *et al.*, 2019).

Con estos antecedentes nos planteamos profundizar en el estudio de la activina en el melanoma y su potencial como biomarcador y diana terapéutica. Nuestros primeros datos confirmaron que la activina es activamente secretada tras la interacción melanoma-macrófago. Para ello realizamos ensayos de co-cultivo con líneas celulares de melanoma y monocitos de sangre periférica, ya que son las células que van a ser reclutadas al tumor para convertirse en TAM (Yang *et al.*, 2018b). Se analizó tanto la proteína secretada al sobrenadante como la expresión de mRNA y se observó que tanto los monocitos/macrófagos como las células tumorales son la fuente de la activina. Los monocitos condicionados por el melanoma también expresaban el perfil pro-tumoral de citoquinas CCL20 / TNF / VEGFA, así como otros factores implicados en el fenotipo inmunosupresor de los TAM, como *IL10*, *IDO1*, *PTGS2*, que codifica la expresión de COX2, y *CD274*, codificante para PD-L1. También observamos que la activina es capaz de inducir la expresión de todos estos genes, incluso de *INHBA*, generando un mecanismo de retroalimentación positiva en los monocitos. Para explorar los efectos de la activina en el condicionamiento de los monocitos por parte del melanoma decidimos utilizar el inhibidor SB-431542, que inhibe la acción de los receptores ALK5, ALK4 y ALK7, impidiendo la señalización a través de SMAD2 (Inman et al., 2002). Observamos que la inducción de estos genes en el co-cultivo era parcialmente revertida en presencia del inhibidor salvo por los genes IL10 y CD274, que no se vieron afectados. Esto sugiere que la activina es un factor intermediario clave en la interacción entre monocitos y macrófagos, ya que se secreta desde el comienzo de la interacción

y además su señalización controla la secreción de otros factores pro-tumorales e inmunosupresores.

En conjunto, estos resultados indican que el condicionamiento de los macrófagos/monocitos por parte del melanoma inicia un proceso de retroalimentación positiva que induce la expresión de activina, así como un conjunto de factores con un papel inmunosupresor, como COX2/PGE_2 (Mao et al., 2013) e IDO1 (Hennequart et al., 2017), de supervivencia como TNF (Smith *et al.*, 2014), pro-invasivos como CCL20 (Samaniego *et al.*, 2018) o pro-angiogénicos como VEGFA (Apte et al., 2019). Además de tener un efecto autocrino, la activina, debido a su gran difusibilidad (McDowell et al., 1997), puede promover efectos locales paracrinos en monocitos y otras células inmunes recién reclutadas. Para evaluar la capacidad inmunosupresora del fenotipo adquirido por los TAM y la contribución de la activina en esta funcionalidad, decidimos realizar un ensayo de inmunosupresión de linfocitos en presencia de medios condicionados obtenidos de los co-cultivos de monocitos y melanoma o en presencia de los monocitos previamente condicionados por el melanoma. Este ensayo se realizó mediante la estimulación de linfocitos T con bolas anti-CD3/CD28 y posterior evaluación de la proliferación mediante la tinción con CFSE (Carboxyfluorescein succinimidyl ester). Como resultado obtuvimos no solo que tanto el medio de cultivo como el monocito condicionado son capaces de frenar la proliferación de los linfocitos, sino que además este efecto inmunosupresor es abolido cuando el condicionamiento tiene lugar en presencia del inhibidor SB-431542. Este resultado apoya la hipótesis de que la activina tiene un papel relevante en el fenotipo inmunosupresor de los macrófagos.

También quisimos corroborar los efectos de la activina sobre la migración y en la invasión tumoral, ya que, son factores clave en la diseminación metastásica (Welch and Hurst, 2019). Ya se había descrito con anterioridad que la activina estimula la migración y la invasión en otros tipos de cáncer (Ries et al., 2020). Sin embargo, en nuestros ensayos de invasión tridimensional observamos que, aunque la activina por sí sola no aumenta la invasión, cuando su señalización es bloqueada en los medios condicionados, las propiedades pro-invasivas de los mismos se inhiben. Esto indica que los efectos de la activina son indirectos, es decir, cuando la activina es producida en el co-cultivo, se induce en el monocito la secreción de ciertas moléculas que promueven la invasión tridimensional del melanoma, tales como PGE_2, CCL20 o TNFα.

Con respecto al uso de la activina como biomarcador, varios estudios han intentado correlacionar la expresión de mRNA o de proteína de la activina con la supervivencia y parámetros clinicopatológicos de pacientes de cáncer. En la gran mayoría de tumores sólidos el mRNA de INHBA está sobre-expresado, y, además, correlaciona con la progresión de la enfermedad y con un mal pronóstico (Antsiferova *et al.*, 2011; Bashir *et al.*, 2015; Donovan *et al.*, 2017; Lonardo et al., 2011). Del mismo modo, altos niveles de expresión de la subunidad βA de la activina también se asociaron con una mala supervivencia en varios tipos de cáncer como adenocarcinoma endometrioide (Mylonas, 2011), carcinoma urotelial (Lee et al., 2015) y leucemia mieloide aguda (Si et al., 2018). En resumen, la evidencia disponible sugiere que la expresión elevada de activina tiene un impacto negativo en la progresión de la enfermedad. Sin embargo, a día de hoy se han realizado pocos estudios confirmatorios en el mismo tipo de cáncer y el beneficio de usar la activina como biomarcador en el ámbito clínico todavía necesita ser testado.

En este trabajo quisimos explorar si los TAM o las células tumorales constituían una fuente importante de activina in vivo. Para ello procedimos a purificar los TAM y las células tumorales de 4 tumores primarios de pacientes en estadio IV para cuantificar la expresión de mRNA. Observamos que tanto los TAM como las células tumorales expresaban INHBA, mientras que los monocitos extraídos de la sangre de los pacientes no, indicando que la secreción de activina es un suceso local que tiene lugar en el microambiente tumoral y no un condicionamiento sistémico de las células mieloides. A continuación, decidimos investigar si la expresión de la activina en el microambiente tumoral del melanoma tenía significancia clínica. Utilizando nuestro propio método de fenotipado mediante microscopía confocal multicolor y cuantificación a nivel de células individuales hemos analizado el contenido de activina en dos cohortes de pacientes con melanoma primario: una primera cohorte de 40 pacientes y una cohorte confirmatoria de 83 pacientes. Nuestros datos indican que la activina expresada tanto por TAM como por células tumorales es un factor pronostico independiente de la progresión metastática en pacientes con melanoma cutáneo. Como nuevo biomarcador del progreso de la enfermedad, la expresión de activina en tumores primarios puede proporcionar una guía para la inmunoterapia adyuvante, ya que así solo aquellos pacientes con alto riesgo de diseminación metastásica y mal pronóstico se

verían beneficiados, aportando más seguridad a los tratamientos y evitando los efectos adversos para aquellos pacientes con menor riesgo (Rizk et al., 2020; Schuchter, 2017).

Por lo tanto, y atendiendo a que la activina controla la inducción de funciones inmunosupreosras y pro-invasivas, consideramos que debería ser valorada como una potencial diana para mejorar los tratamientos de inmunoterapia. Para probar esta hipótesis decidimos utilizar un modelo de terapia celular adoptiva (ACT, por sus siglas en inglés "adoptive cell transfer") con linfocitos humanos en ratones inmunodeficientes inoculados con un melanoma humano, ya descrito anteriormente (Hennequart *et al.*, 2017; Sanmamed et al., 2015). En primer lugar, observamos como la terapia con células T era capaz de controlar el crecimiento de los tumores de melanoma (rechazo alogénico). Sin embargo, cuando el tumor inyectado era mezclado con monocitos, el efecto anti-tumoral de la ACT se perdía, sugiriendo que los monocitos/macrófagos presentes en el tumor suprimían el rechazo alogénico y bloqueaban la respuesta de la ACT. Curiosamente, la respuesta anti-tumoral se recuperaba mediante el bloqueo de la activina en los *xenograft* que presentaban mezcla de monocitos y células tumorales, sin alterar la progresión tumoral de aquellos que solo tenían células de melanoma. Así pues, parece que bloquear la activina podría complementar a la inmunoterapia para aliviar el microambiente inmunosupresor creado por las interacciones entre el melanoma y lo monocitos.

En conclusión, nuestros resultados apoyan la hipótesis de que la activina es una citoquina con un papel decisivo en la orquestación del microambiente pro-tumoral e inmunosupresor del melanoma humano. Además, cabe destacar que el bloqueo de la activina podría contribuir a estimular la respuesta inmune anti-tumoral y su combinación con inmunoterapia podría ser un tratamiento muy prometedor tanto en melanoma como en otros tipos de cáncer.

CONCLUSIONES

Los resultados presentados en esta tesis apoyan las siguientes conclusiones:

1. No se ha observado una correlación entre la evolución de pacientes y el tamaño, densidad y estado de polarización de los macrófagos asociados a tumor (TAM) de melanoma primario, en dos colecciones independientes de melanoma humano.

2. Los niveles de expresión proteica de las citoquinas CCL20/TNF/VEGFA por los TAM, cuantificados en los melanomas primarios mediante IF-multicolor a nivel de célula única, correlaciona, de forma independiente, con un mal pronóstico en pacientes de melanoma en estadios I-III en dos series independientes de pacientes.

3. La expresión del perfil pro-tumoral de citoquinas CCL20/TNF/VEGFA en TAM tiene lugar principalmente a través de las vías de señalización de p53 y NF-κB.

4. La activina A tiene un papel intermediario clave en el condicionamiento de los monocitos /macrófagos por parte del melanoma *in vitro* y promueve la adquisición de un perfil pro-tumoral e inmunosupresor en los monocitos condicionados por melanoma.

5. El nivel alto de expresión de activina A tanto en TAM como en células tumorales correlaciona, de forma independiente, con un mal pronóstico en pacientes de melanoma en estadios I-III, en dos colecciones independientes de melanoma humano.

6. La activina A confiere resistencia a la respuesta anti-tumoral en un modelo de *xenograft* de melanoma en ratones inmunodeficientes tratados con terapia celular adoptiva (ACT). Así, la activina se muestra como una potencial diana terapéutica para el tratamiento de melanoma y otros tipos de cáncer.

BIBLIOGRAFÍA

Alexandrov, L.B., Nik-Zainal, S., Wedge, D.C., Aparicio, S.A., Behjati, S., Biankin, A.V., Bignell, G.R., Bolli, N., Borg, A., Borresen-Dale, A.L., et al. (2013). Signatures of mutational processes in human cancer. Nature *500*, 415-421. 10.1038/nature12477.

Alkasalias, T., Moyano-Galceran, L., Arsenian-Henriksson, M., and Lehti, K. (2018). Fibroblasts in the Tumor Microenvironment: Shield or Spear? Int J Mol Sci *19*. 10.3390/ijms19051532.

Antsiferova, M., Huber, M., Meyer, M., Piwko-Czuchra, A., Ramadan, T., MacLeod, A.S., Havran, W.L., Dummer, R., Hohl, D., and Werner, S. (2011). Activin enhances skin tumourigenesis and malignant progression by inducing a pro-tumourigenic immune cell response. Nat Commun *2*, 576. 10.1038/ncomms1585.

Antsiferova, M., Piwko-Czuchra, A., Cangkrama, M., Wietecha, M., Sahin, D., Birkner, K., Amann, V.C., Levesque, M., Hohl, D., Dummer, R., and Werner, S. (2017). Activin promotes skin carcinogenesis by attraction and reprogramming of macrophages. EMBO Mol Med *9*, 27-45. 10.15252/emmm.201606493.

Antsiferova, M., and Werner, S. (2012). The bright and the dark sides of activin in wound healing and cancer. J Cell Sci *125*, 3929-3937. 10.1242/jcs.094789.

Apte, R.S., Chen, D.S., and Ferrara, N. (2019). VEGF in Signaling and Disease: Beyond Discovery and Development. Cell *176*, 1248-1264. 10.1016/j.cell.2019.01.021.

Arnold, M., de Vries, E., Whiteman, D.C., Jemal, A., Bray, F., Parkin, D.M., and Soerjomataram, I. (2018). Global burden of cutaneous melanoma attributable to ultraviolet radiation in 2012. Int J Cancer *143*, 1305-1314. 10.1002/ijc.31527.

Balch, C.M., Gershenwald, J.E., Soong, S.J., Thompson, J.F., Atkins, M.B., Byrd, D.R., Buzaid, A.C., Cochran, A.J., Coit, D.G., Ding, S., et al. (2009). Final version of 2009 AJCC melanoma staging and classification. J Clin Oncol *27*, 6199-6206. 10.1200/JCO.2009.23.4799.

Balch, C.M., Gershenwald, J.E., Soong, S.J., Thompson, J.F., Ding, S., Byrd, D.R., Cascinelli, N., Cochran, A.J., Coit, D.G., Eggermont, A.M., et al. (2010). Multivariate analysis of prognostic

factors among 2,313 patients with stage III melanoma: comparison of nodal micrometastases versus macrometastases. J Clin Oncol *28*, 2452-2459. 10.1200/JCO.2009.27.1627.

Balkwill, F. (2006). TNF-alpha in promotion and progression of cancer. Cancer Metastasis Rev *25*, 409-416. 10.1007/s10555-006-9005-3.

Barros, M.H., Hauck, F., Dreyer, J.H., Kempkes, B., and Niedobitek, G. (2013). Macrophage polarisation: an immunohistochemical approach for identifying M1 and M2 macrophages. PLoS One *8*, e80908. 10.1371/journal.pone.0080908.

Bashir, M., Damineni, S., Mukherjee, G., and Kondaiah, P. (2015). Activin-A signaling promotes epithelial-mesenchymal transition, invasion, and metastatic growth of breast cancer. NPJ Breast Cancer *1*, 15007. 10.1038/npjbcancer.2015.7.

Batlle, E., and Massague, J. (2019). Transforming Growth Factor-beta Signaling in Immunity and Cancer. Immunity *50*, 924-940. 10.1016/j.immuni.2019.03.024.

Bellomo, C., Caja, L., and Moustakas, A. (2016). Transforming growth factor beta as regulator of cancer stemness and metastasis. Br J Cancer *115*, 761-769. 10.1038/bjc.2016.255.

Biswas, S.K., Chittezhath, M., Shalova, I.N., and Lim, J.Y. (2012). Macrophage polarization and plasticity in health and disease. Immunol Res *53*, 11-24. 10.1007/s12026-012-8291-9.

Biswas, S.K., and Mantovani, A. (2010). Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. Nat Immunol *11*, 889-896. 10.1038/ni.1937.

Bronte, V., Brandau, S., Chen, S.H., Colombo, M.P., Frey, A.B., Greten, T.F., Mandruzzato, S., Murray, P.J., Ochoa, A., Ostrand-Rosenberg, S., et al. (2016). Recommendations for myeloid-derived suppressor cell nomenclature and characterization standards. Nat Commun *7*, 12150. 10.1038/ncomms12150.

Burdette, J.E., Jeruss, J.S., Kurley, S.J., Lee, E.J., and Woodruff, T.K. (2005). Activin A mediates growth inhibition and cell cycle arrest through Smads in human breast cancer cells. Cancer Res *65*, 7968-7975. 10.1158/0008-5472.CAN-04-3553.

Chang, A.L., Miska, J., Wainwright, D.A., Dey, M., Rivetta, C.V., Yu, D., Kanojia, D., Pituch, K.C., Qiao, J., Pytel, P., et al. (2016). CCL2 Produced by the Glioma Microenvironment Is Essential for the Recruitment of Regulatory T Cells and Myeloid-Derived Suppressor Cells. Cancer Res *76*, 5671-5682. 10.1158/0008-5472.CAN-16-0144.

Chang, K.P., Kao, H.K., Liang, Y., Cheng, M.H., Chang, Y.L., Liu, S.C., Lin, Y.C., Ko, T.Y., Lee, Y.S., Tsai, C.L., et al. (2010). Overexpression of activin A in oral squamous cell carcinoma: association with poor prognosis and tumor progression. Ann Surg Oncol *17*, 1945-1956. 10.1245/s10434-010-0926-2.

Chen, E.P., Markosyan, N., Connolly, E., Lawson, J.A., Li, X., Grant, G.R., Grosser, T., FitzGerald, G.A., and Smyth, E.M. (2014). Myeloid Cell COX-2 deletion reduces mammary tumor growth through enhanced cytotoxic T-lymphocyte function. Carcinogenesis *35*, 1788-1797. 10.1093/carcin/bgu053.

Chen, W., and Ten Dijke, P. (2016). Immunoregulation by members of the TGFbeta superfamily. Nat Rev Immunol *16*, 723-740. 10.1038/nri.2016.112.

Chen, W., Woodruff, T.K., and Mayo, K.E. (2000). Activin A-induced HepG2 liver cell apoptosis: involvement of activin receptors and smad proteins. Endocrinology *141*, 1263-1272. 10.1210/endo.141.3.7361.

Chevrier, S., Levine, J.H., Zanotelli, V.R.T., Silina, K., Schulz, D., Bacac, M., Ries, C.H., Ailles, L., Jewett, M.A.S., Moch, H., et al. (2017). An Immune Atlas of Clear Cell Renal Cell Carcinoma. Cell *169*, 736-749 e718. 10.1016/j.cell.2017.04.016.

Chittezhath, M., Dhillon, M.K., Lim, J.Y., Laoui, D., Shalova, I.N., Teo, Y.L., Chen, J., Kamaraj, R., Raman, L., Lum, J., et al. (2014). Molecular profiling reveals a tumor-promoting phenotype of monocytes and macrophages in human cancer progression. Immunity *41*, 815-829. 10.1016/j.immuni.2014.09.014.

Coffelt, S.B., Wellenstein, M.D., and de Visser, K.E. (2016). Neutrophils in cancer: neutral no more. Nat Rev Cancer *16*, 431-446. 10.1038/nrc.2016.52.

Coronella, J.A., Telleman, P., Kingsbury, G.A., Truong, T.D., Hays, S., and Junghans, R.P. (2001). Evidence for an antigen-driven humoral immune response in medullary ductal breast cancer. Cancer Res *61*, 7889-7899.

Curti, B.D., and Faries, M.B. (2021). Recent Advances in the Treatment of Melanoma. N Engl J Med *384*, 2229-2240. 10.1056/NEJMra2034861.

Cuschieri, J., Bulger, E., Grinsell, R., Garcia, I., and Maier, R.V. (2008). Insulin regulates macrophage activation through activin A. Shock *29*, 285-290. 10.1097/SHK.0b013e318123e4d0.

Dalkin, A.C., Gilrain, J.T., Bradshaw, D., and Myers, C.E. (1996). Activin inhibition of prostate cancer cell growth: selective actions on androgen-responsive LNCaP cells. Endocrinology *137*, 5230-5235. 10.1210/endo.137.12.8940339.

Damsky, W.E., Theodosakis, N., and Bosenberg, M. (2014). Melanoma metastasis: new concepts and evolving paradigms. Oncogene *33*, 2413-2422. 10.1038/onc.2013.194.

de Guise, C., Lacerte, A., Rafiei, S., Reynaud, R., Roy, M., Brue, T., and Lebrun, J.J. (2006). Activin inhibits the human Pit-1 gene promoter through the p38 kinase pathway in a Smad-independent manner. Endocrinology *147*, 4351-4362. 10.1210/en.2006-0444.

Donovan, P., Dubey, O.A., Kallioinen, S., Rogers, K.W., Muehlethaler, K., Muller, P., Rimoldi, D., and Constam, D.B. (2017). Paracrine Activin-A Signaling Promotes Melanoma Growth and Metastasis through Immune Evasion. J Invest Dermatol *137*, 2578-2587. 10.1016/j.jid.2017.07.845.

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

Elliott, R.L., and Blobe, G.C. (2005). Role of transforming growth factor Beta in human cancer. J Clin Oncol *23*, 2078-2093. 10.1200/JCO.2005.02.047.

Feng, X.H., and Derynck, R. (2005). Specificity and versatility in tgf-beta signaling through Smads. Annu Rev Cell Dev Biol *21*, 659-693. 10.1146/annurev.cellbio.21.022404.142018.

Franklin, R.A., and Li, M.O. (2016). Ontogeny of Tumor-associated Macrophages and Its Implication in Cancer Regulation. Trends Cancer *2*, 20-34. 10.1016/j.trecan.2015.11.004.

Fridman, W.H., Pages, F., Sautes-Fridman, C., and Galon, J. (2012). The immune contexture in human tumours: impact on clinical outcome. Nat Rev Cancer *12*, 298-306. 10.1038/nrc3245.

Fu, C., and Jiang, A. (2018). Dendritic Cells and CD8 T Cell Immunity in Tumor Microenvironment. Front Immunol *9*, 3059. 10.3389/fimmu.2018.03059.

Gabrilovich, D.I. (2017). Myeloid-Derived Suppressor Cells. Cancer Immunol Res *5*, 3-8. 10.1158/2326-6066.CIR-16-0297.

Galon, J., Fox, B.A., Bifulco, C.B., Masucci, G., Rau, T., Botti, G., Marincola, F.M., Ciliberto, G., Pages, F., Ascierto, P.A., and Capone, M. (2016). Immunoscore and Immunoprofiling in cancer: an update from the melanoma and immunotherapy bridge 2015. J Transl Med *14*, 273. 10.1186/s12967-016-1029-z.

Gazzaniga, S., Bravo, A.I., Guglielmotti, A., van Rooijen, N., Maschi, F., Vecchi, A., Mantovani, A., Mordoh, J., and Wainstok, R. (2007). Targeting tumor-associated macrophages and inhibition of MCP-1 reduce angiogenesis and tumor growth in a human melanoma xenograft. J Invest Dermatol *127*, 2031-2041. 10.1038/sj.jid.5700827.

Ginhoux, F., and Guilliams, M. (2016). Tissue-Resident Macrophage Ontogeny and Homeostasis. Immunity *44*, 439-449. 10.1016/j.immuni.2016.02.024.

Grivennikov, S.I., Greten, F.R., and Karin, M. (2010). Immunity, inflammation, and cancer. Cell *140*, 883-899. 10.1016/j.cell.2010.01.025.

Guerriero, J.L. (2018). Macrophages: The Road Less Traveled, Changing Anticancer Therapy. Trends Mol Med *24*, 472-489. 10.1016/j.molmed.2018.03.006.

Gutierrez-Gonzalez, A., Martinez-Moreno, M., Samaniego, R., Arellano-Sanchez, N., Salinas-Munoz, L., Relloso, M., Valeri, A., Martinez-Lopez, J., Corbi, A.L., Hidalgo, A., et al. (2016).

Evaluation of the potential therapeutic benefits of macrophage reprogramming in multiple myeloma. Blood *128*, 2241-2252. 10.1182/blood-2016-01-695395.

Hagemann, T., Lawrence, T., McNeish, I., Charles, K.A., Kulbe, H., Thompson, R.G., Robinson, S.C., and Balkwill, F.R. (2008). "Re-educating" tumor-associated macrophages by targeting NF-kappaB. J Exp Med *205*, 1261-1268. 10.1084/jem.20080108.

Halin, S., Rudolfsson, S.H., Van Rooijen, N., and Bergh, A. (2009). Extratumoral macrophages promote tumor and vascular growth in an orthotopic rat prostate tumor model. Neoplasia *11*, 177-186. 10.1593/neo.81338.

Hanahan, D., and Weinberg, R.A. (2011). Hallmarks of cancer: the next generation. Cell *144*, 646-674. 10.1016/j.cell.2011.02.013.

Hedger, M.P., and de Kretser, D.M. (2013). The activins and their binding protein, follistatin-Diagnostic and therapeutic targets in inflammatory disease and fibrosis. Cytokine Growth Factor Rev *24*, 285-295. 10.1016/j.cytogfr.2013.03.003.

Hedger, M.P., Drummond, A.E., Robertson, D.M., Risbridger, G.P., and de Kretser, D.M. (1989). Inhibin and activin regulate [3H]thymidine uptake by rat thymocytes and 3T3 cells in vitro. Mol Cell Endocrinol *61*, 133-138. 10.1016/0303-7207(89)90198-6.

Heinz, M., Niederleithner, H.L., Puujalka, E., Soler-Cardona, A., Grusch, M., Pehamberger, H., Loewe, R., and Petzelbauer, P. (2015). Activin A is anti-lymphangiogenic in a melanoma mouse model. J Invest Dermatol *135*, 212-221. 10.1038/jid.2014.328.

Heldin, C.H., and Moustakas, A. (2016). Signaling Receptors for TGF-beta Family Members. Cold Spring Harb Perspect Biol *8*. 10.1101/cshperspect.a022053.

Hennequart, M., Pilotte, L., Cane, S., Hoffmann, D., Stroobant, V., Plaen, E., and Van den Eynde, B.J. (2017). Constitutive IDO1 Expression in Human Tumors Is Driven by Cyclooxygenase-2 and Mediates Intrinsic Immune Resistance. Cancer Immunol Res *5*, 695-709. 10.1158/2326-6066.CIR-16-0400.

Heusinkveld, M., de Vos van Steenwijk, P.J., Goedemans, R., Ramwadhdoebe, T.H., Gorter, A., Welters, M.J., van Hall, T., and van der Burg, S.H. (2011). M2 macrophages induced by prostaglandin E2 and IL-6 from cervical carcinoma are switched to activated M1 macrophages by CD4+ Th1 cells. J Immunol *187*, 1157-1165. 10.4049/jimmunol.1100889.

Hiraoka, K., Miyamoto, M., Cho, Y., Suzuoki, M., Oshikiri, T., Nakakubo, Y., Itoh, T., Ohbuchi, T., Kondo, S., and Katoh, H. (2006). Concurrent infiltration by CD8+ T cells and CD4+ T cells is a favourable prognostic factor in non-small-cell lung carcinoma. Br J Cancer *94*, 275-280. 10.1038/sj.bjc.6602934.

Hoda, M.A., Munzker, J., Ghanim, B., Schelch, K., Klikovits, T., Laszlo, V., Sahin, E., Bedeir, A., Lackner, A., Dome, B., et al. (2012). Suppression of activin A signals inhibits growth of malignant pleural mesothelioma cells. Br J Cancer *107*, 1978-1986. 10.1038/bjc.2012.519.

Hoek, K.S., Schlegel, N.C., Brafford, P., Sucker, A., Ugurel, S., Kumar, R., Weber, B.L., Nathanson, K.L., Phillips, D.J., Herlyn, M., et al. (2006). Metastatic potential of melanomas defined by specific gene expression profiles with no BRAF signature. Pigment Cell Res *19*, 290-302. 10.1111/j.1600-0749.2006.00322.x.

Hsieh, C.S., Lee, H.M., and Lio, C.W. (2012). Selection of regulatory T cells in the thymus. Nat Rev Immunol *12*, 157-167. 10.1038/nri3155.

Hyuga, S., Kawasaki, N., Hashimoto, O., Hyuga, M., Ohta, M., Yamagata, S., Yamagata, T., and Hayakawa, T. (2000). Possible role of hepatocyte growth factor/scatter factor and activin A produced by the target organ in liver metastasis. Cancer Lett *153*, 137-143. 10.1016/s0304-3835(00)00360-8.

Inman, G.J., Nicolas, F.J., Callahan, J.F., Harling, J.D., Gaster, L.M., Reith, A.D., Laping, N.J., and Hill, C.S. (2002). SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. Mol Pharmacol *62*, 65-74. 10.1124/mol.62.1.65.

Jacquelot, N., Roberti, M.P., Enot, D.P., Rusakiewicz, S., Ternes, N., Jegou, S., Woods, D.M., Sodre, A.L., Hansen, M., Meirow, Y., et al. (2017). Predictors of responses to immune checkpoint blockade in advanced melanoma. Nat Commun *8*, 592. 10.1038/s41467-017-00608-2.

Jensen, T.O., Schmidt, H., Moller, H.J., Hoyer, M., Maniecki, M.B., Sjoegren, P., Christensen, I.J., and Steiniche, T. (2009). Macrophage markers in serum and tumor have prognostic impact in American Joint Committee on Cancer stage I/II melanoma. J Clin Oncol *27*, 3330-3337. 10.1200/JCO.2008.19.9919.

Jiang, L., Si, T., Yu, M., Zeng, X., Morse, H.C., 3rd, Lu, Y., Ouyang, G., and Zhou, J.X. (2018). The tumor suppressive role of inhibin betaA in diffuse large B-cell lymphoma. Leuk Lymphoma *59*, 1202-1212. 10.1080/10428194.2017.1372574.

Jinushi, M., Chiba, S., Yoshiyama, H., Masutomi, K., Kinoshita, I., Dosaka-Akita, H., Yagita, H., Takaoka, A., and Tahara, H. (2011). Tumor-associated macrophages regulate tumorigenicity and anticancer drug responses of cancer stem/initiating cells. Proc Natl Acad Sci U S A *108*, 12425-12430. 10.1073/pnas.1106645108.

Jung, B.H., Beck, S.E., Cabral, J., Chau, E., Cabrera, B.L., Fiorino, A., Smith, E.J., Bocanegra, M., and Carethers, J.M. (2007). Activin type 2 receptor restoration in MSI-H colon cancer suppresses growth and enhances migration with activin. Gastroenterology *132*, 633-644. 10.1053/j.gastro.2006.11.018.

Kalli, M., Mpekris, F., Wong, C.K., Panagi, M., Ozturk, S., Thiagalingam, S., Stylianopoulos, T., and Papageorgis, P. (2019). Activin A Signaling Regulates IL13Ralpha2 Expression to Promote Breast Cancer Metastasis. Front Oncol *9*, 32. 10.3389/fonc.2019.00032.

Kalluri, R. (2016). The biology and function of fibroblasts in cancer. Nat Rev Cancer *16*, 582-598. 10.1038/nrc.2016.73.

Kaneda, H., Arao, T., Matsumoto, K., De Velasco, M.A., Tamura, D., Aomatsu, K., Kudo, K., Sakai, K., Nagai, T., Fujita, Y., et al. (2011). Activin A inhibits vascular endothelial cell growth and

suppresses tumour angiogenesis in gastric cancer. Br J Cancer *105*, 1210-1217. 10.1038/bjc.2011.348.

Lahmar, Q., Keirsse, J., Laoui, D., Movahedi, K., Van Overmeire, E., and Van Ginderachter, J.A. (2016). Tissue-resident versus monocyte-derived macrophages in the tumor microenvironment. Biochim Biophys Acta *1865*, 23-34. 10.1016/j.bbcan.2015.06.009.

Lavin, Y., Kobayashi, S., Leader, A., Amir, E.D., Elefant, N., Bigenwald, C., Remark, R., Sweeney, R., Becker, C.D., Levine, J.H., et al. (2017). Innate Immune Landscape in Early Lung Adenocarcinoma by Paired Single-Cell Analyses. Cell *169*, 750-765 e717. 10.1016/j.cell.2017.04.014.

Lee, H.Y., Li, C.C., Huang, C.N., Li, W.M., Yeh, H.C., Ke, H.L., Yang, K.F., Liang, P.I., Li, C.F., and Wu, W.J. (2015). INHBA overexpression indicates poor prognosis in urothelial carcinoma of urinary bladder and upper tract. J Surg Oncol *111*, 414-422. 10.1002/jso.23836.

Leivonen, S.K., and Kahari, V.M. (2007). Transforming growth factor-beta signaling in cancer invasion and metastasis. Int J Cancer *121*, 2119-2124. 10.1002/ijc.23113.

Li, C., Zhang, N., Zhou, J., Ding, C., Jin, Y., Cui, X., Pu, K., and Zhu, Y. (2018). Peptide Blocking of PD-1/PD-L1 Interaction for Cancer Immunotherapy. Cancer Immunol Res *6*, 178-188. 10.1158/2326-6066.CIR-17-0035.

Li, D., Duan, M., Feng, Y., Geng, L., Li, X., and Zhang, W. (2016). MiR-146a modulates macrophage polarization in systemic juvenile idiopathic arthritis by targeting INHBA. Mol Immunol *77*, 205-212. 10.1016/j.molimm.2016.08.007.

Liu, Y., Li, P.K., Li, C., and Lin, J. (2010). Inhibition of STAT3 signaling blocks the anti-apoptotic activity of IL-6 in human liver cancer cells. J Biol Chem *285*, 27429-27439. 10.1074/jbc.M110.142752.

Locati, M., Curtale, G., and Mantovani, A. (2020). Diversity, Mechanisms, and Significance of Macrophage Plasticity. Annu Rev Pathol *15*, 123-147. 10.1146/annurev-pathmechdis-012418-012718.

Lonardo, E., Hermann, P.C., Mueller, M.T., Huber, S., Balic, A., Miranda-Lorenzo, I., Zagorac, S., Alcala, S., Rodriguez-Arabaolaza, I., Ramirez, J.C., et al. (2011). Nodal/Activin signaling drives self-renewal and tumorigenicity of pancreatic cancer stem cells and provides a target for combined drug therapy. Cell Stem Cell *9*, 433-446. 10.1016/j.stem.2011.10.001.

Lowe, J.M., Menendez, D., Bushel, P.R., Shatz, M., Kirk, E.L., Troester, M.A., Garantziotis, S., Fessler, M.B., and Resnick, M.A. (2014). p53 and NF-kappaB coregulate proinflammatory gene responses in human macrophages. Cancer Res *74*, 2182-2192. 10.1158/0008-5472.CAN-13-1070.

Lowry, L.E., and Zehring, W.A. (2017). Potentiation of Natural Killer Cells for Cancer Immunotherapy: A Review of Literature. Front Immunol *8*, 1061. 10.3389/fimmu.2017.01061.

Mantovani, A., Allavena, P., Sica, A., and Balkwill, F. (2008). Cancer-related inflammation. Nature *454*, 436-444. 10.1038/nature07205.

Mantovani, A., Schioppa, T., Porta, C., Allavena, P., and Sica, A. (2006). Role of tumor-associated macrophages in tumor progression and invasion. Cancer Metastasis Rev *25*, 315-322. 10.1007/s10555-006-9001-7.

Mantovani, A., Sozzani, S., Locati, M., Allavena, P., and Sica, A. (2002). Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol *23*, 549-555. 10.1016/s1471-4906(02)02302-5.

Martello, G., Zacchigna, L., Inui, M., Montagner, M., Adorno, M., Mamidi, A., Morsut, L., Soligo, S., Tran, U., Dupont, S., et al. (2007). MicroRNA control of Nodal signalling. Nature *449*, 183-188. 10.1038/nature06100.

Massague, J., and Chen, Y.G. (2000). Controlling TGF-beta signaling. Genes Dev *14*, 627-644.

McDowell, N., Zorn, A.M., Crease, D.J., and Gurdon, J.B. (1997). Activin has direct long-range signalling activity and can form a concentration gradient by diffusion. Curr Biol *7*, 671-681. 10.1016/s0960-9822(06)00294-6.

McGovern, N., Schlitzer, A., Gunawan, M., Jardine, L., Shin, A., Poyner, E., Green, K., Dickinson, R., Wang, X.N., Low, D., et al. (2014). Human dermal CD14(+) cells are a transient population of monocyte-derived macrophages. Immunity *41*, 465-477. 10.1016/j.immuni.2014.08.006.

Miller, A.J., and Mihm, M.C., Jr. (2006). Melanoma. N Engl J Med *355*, 51-65. 10.1056/NEJMra052166.

Milne, K., Kobel, M., Kalloger, S.E., Barnes, R.O., Gao, D., Gilks, C.B., Watson, P.H., and Nelson, B.H. (2009). Systematic analysis of immune infiltrates in high-grade serous ovarian cancer reveals CD20, FoxP3 and TIA-1 as positive prognostic factors. PLoS One *4*, e6412. 10.1371/journal.pone.0006412.

Morianos, I., Papadopoulou, G., Semitekolou, M., and Xanthou, G. (2019). Activin-A in the regulation of immunity in health and disease. J Autoimmun *104*, 102314. 10.1016/j.jaut.2019.102314.

Movahedi, K., Laoui, D., Gysemans, C., Baeten, M., Stange, G., Van den Bossche, J., Mack, M., Pipeleers, D., In't Veld, P., De Baetselier, P., and Van Ginderachter, J.A. (2010). Different tumor microenvironments contain functionally distinct subsets of macrophages derived from Ly6C(high) monocytes. Cancer Res *70*, 5728-5739. 10.1158/0008-5472.CAN-09-4672.

Mylonas, I. (2011). Inhibin-alpha subunit expression in uterine endometrioid adenocarcinomas and endometrial cancer cell lines: a potential prognostic factor. Int J Mol Med *27*, 309-318. 10.3892/ijmm.2010.586.

Namwanje, M., and Brown, C.W. (2016). Activins and Inhibins: Roles in Development, Physiology, and Disease. Cold Spring Harb Perspect Biol *8*. 10.1101/cshperspect.a021881.

Ni, X., Tao, J., Barbi, J., Chen, Q., Park, B.V., Li, Z., Zhang, N., Lebid, A., Ramaswamy, A., Wei, P., et al. (2018). YAP Is Essential for Treg-Mediated Suppression of Antitumor Immunity. Cancer Discov *8*, 1026-1043. 10.1158/2159-8290.CD-17-1124.

Nishikawa, H., and Sakaguchi, S. (2010). Regulatory T cells in tumor immunity. Int J Cancer *127*, 759-767. 10.1002/ijc.25429.

Nomura, M., Tanaka, K., Wang, L., Goto, Y., Mukasa, C., Ashida, K., and Takayanagi, R. (2013). Activin type IB receptor signaling in prostate cancer cells promotes lymph node metastasis in a xenograft model. Biochem Biophys Res Commun *430*, 340-346. 10.1016/j.bbrc.2012.11.011.

Nusing, R.M., and Barsig, J. (1999). Induction of prostanoid, nitric oxide, and cytokine formation in rat bone marrow derived macrophages by activin A. Br J Pharmacol *127*, 919-926. 10.1038/sj.bjp.0702626.

Nusing, R.M., Mohr, S., and Ullrich, V. (1995). Activin A and retinoic acid synergize in cyclooxygenase-1 and thromboxane synthase induction during differentiation of J774.1 macrophages. Eur J Biochem *227*, 130-136. 10.1111/j.1432-1033.1995.tb20368.x.

O'Keeffe, M., Mok, W.H., and Radford, K.J. (2015). Human dendritic cell subsets and function in health and disease. Cell Mol Life Sci *72*, 4309-4325. 10.1007/s00018-015-2005-0.

Ohga, E., Matsuse, T., Teramoto, S., Katayama, H., Nagase, T., Fukuchi, Y., and Ouchi, Y. (1996). Effects of activin A on proliferation and differentiation of human lung fibroblasts. Biochem Biophys Res Commun *228*, 391-396. 10.1006/bbrc.1996.1672.

Ott, P.A., Hu, Z., Keskin, D.B., Shukla, S.A., Sun, J., Bozym, D.J., Zhang, W., Luoma, A., Giobbie-Hurder, A., Peter, L., et al. (2017). An immunogenic personal neoantigen vaccine for patients with melanoma. Nature *547*, 217-221. 10.1038/nature22991.

Pages, F., Berger, A., Camus, M., Sanchez-Cabo, F., Costes, A., Molidor, R., Mlecnik, B., Kirilovsky, A., Nilsson, M., Damotte, D., et al. (2005). Effector memory T cells, early metastasis, and survival in colorectal cancer. N Engl J Med *353*, 2654-2666. 10.1056/NEJMoa051424.

Panopoulou, E., Murphy, C., Rasmussen, H., Bagli, E., Rofstad, E.K., and Fotsis, T. (2005). Activin A suppresses neuroblastoma xenograft tumor growth via antimitotic and antiangiogenic mechanisms. Cancer Res *65*, 1877-1886. 10.1158/0008-5472.CAN-04-2828.

Perrot, C.Y., Javelaud, D., and Mauviel, A. (2013). Insights into the Transforming Growth Factor-beta Signaling Pathway in Cutaneous Melanoma. Ann Dermatol *25*, 135-144. 10.5021/ad.2013.25.2.135.

Porta, C., Rimoldi, M., Raes, G., Brys, L., Ghezzi, P., Di Liberto, D., Dieli, F., Ghisletti, S., Natoli, G., De Baetselier, P., et al. (2009). Tolerance and M2 (alternative) macrophage polarization are related processes orchestrated by p50 nuclear factor kappaB. Proc Natl Acad Sci U S A *106*, 14978-14983. 10.1073/pnas.0809784106.

Postow, M.A., Sidlow, R., and Hellmann, M.D. (2018). Immune-Related Adverse Events Associated with Immune Checkpoint Blockade. N Engl J Med *378*, 158-168. 10.1056/NEJMra1703481.

Prima, V., Kaliberova, L.N., Kaliberov, S., Curiel, D.T., and Kusmartsev, S. (2017). COX2/mPGES1/PGE2 pathway regulates PD-L1 expression in tumor-associated macrophages and myeloid-derived suppressor cells. Proc Natl Acad Sci U S A *114*, 1117-1122. 10.1073/pnas.1612920114.

Puig-Kroger, A., Sierra-Filardi, E., Dominguez-Soto, A., Samaniego, R., Corcuera, M.T., Gomez-Aguado, F., Ratnam, M., Sanchez-Mateos, P., and Corbi, A.L. (2009). Folate receptor beta is expressed by tumor-associated macrophages and constitutes a marker for M2 anti-inflammatory/regulatory macrophages. Cancer Res *69*, 9395-9403. 10.1158/0008-5472.CAN-09-2050.

Qian, B.Z., and Pollard, J.W. (2010). Macrophage diversity enhances tumor progression and metastasis. Cell *141*, 39-51. 10.1016/j.cell.2010.03.014.

Quail, D.F., and Joyce, J.A. (2013). Microenvironmental regulation of tumor progression and metastasis. Nat Med *19*, 1423-1437. 10.1038/nm.3394.

Rastrelli, M., Tropea, S., Rossi, C.R., and Alaibac, M. (2014). Melanoma: epidemiology, risk factors, pathogenesis, diagnosis and classification. In Vivo *28*, 1005-1011.

Rautela, J., Dagley, L.F., de Oliveira, C.C., Schuster, I.S., Hediyeh-Zadeh, S., Delconte, R.B., Cursons, J., Hennessy, R., Hutchinson, D.S., Harrison, C., et al. (2019). Therapeutic blockade of activin-A improves NK cell function and antitumor immunity. Sci Signal *12*. 10.1126/scisignal.aat7527.

Razanajaona, D., Joguet, S., Ay, A.S., Treilleux, I., Goddard-Leon, S., Bartholin, L., and Rimokh, R. (2007). Silencing of FLRG, an antagonist of activin, inhibits human breast tumor cell growth. Cancer Res *67*, 7223-7229. 10.1158/0008-5472.CAN-07-0805.

REDECAN. Red Española de Registros de Cáncer. [último acceso el 19/12/2022]. Disponible en: https://redecan.org/es/registros-de-cancer.

Ribas, A., and Wolchok, J.D. (2018). Cancer immunotherapy using checkpoint blockade. Science *359*, 1350-1355. 10.1126/science.aar4060.

Ries, A., Schelch, K., Falch, D., Pany, L., Hoda, M.A., and Grusch, M. (2020). Activin A: an emerging target for improving cancer treatment? Expert Opin Ther Targets *24*, 985-996. 10.1080/14728222.2020.1799350.

Rizk, E.M., Seffens, A.M., Trager, M.H., Moore, M.R., Geskin, L.J., Gartrell-Corrado, R.D., Wong, W., and Saenger, Y.M. (2020). Biomarkers Predictive of Survival and Response to Immune Checkpoint Inhibitors in Melanoma. Am J Clin Dermatol *21*, 1-11. 10.1007/s40257-019-00475-1.

Ruffell, B., Chang-Strachan, D., Chan, V., Rosenbusch, A., Ho, C.M., Pryer, N., Daniel, D., Hwang, E.S., Rugo, H.S., and Coussens, L.M. (2014). Macrophage IL-10 blocks CD8+ T cell-dependent responses to chemotherapy by suppressing IL-12 expression in intratumoral dendritic cells. Cancer Cell *26*, 623-637. 10.1016/j.ccell.2014.09.006.

Russick, J., Joubert, P.E., Gillard-Bocquet, M., Torset, C., Meylan, M., Petitprez, F., Dragon-Durey, M.A., Marmier, S., Varthaman, A., Josseaume, N., et al. (2020). Natural killer cells in the human lung tumor microenvironment display immune inhibitory functions. J Immunother Cancer *8*. 10.1136/jitc-2020-001054.

Samaniego, R., Estecha, A., Relloso, M., Longo, N., Escat, J.L., Longo-Imedio, I., Aviles, J.A., del Pozo, M.A., Puig-Kroger, A., and Sanchez-Mateos, P. (2013). Mesenchymal contribution to recruitment, infiltration, and positioning of leukocytes in human melanoma tissues. J Invest Dermatol *133*, 2255-2264. 10.1038/jid.2013.88.

Samaniego, R., Gutierrez-Gonzalez, A., Gutierrez-Seijo, A., Sanchez-Gregorio, S., Garcia-Gimenez, J., Mercader, E., Marquez-Rodas, I., Aviles, J.A., Relloso, M., and Sanchez-Mateos, P. (2018). CCL20 Expression by Tumor-Associated Macrophages Predicts Progression of Human Primary Cutaneous Melanoma. Cancer Immunol Res *6*, 267-275. 10.1158/2326-6066.CIR-17-0198.

Samaniego, R., Palacios, B.S., Domiguez-Soto, A., Vidal, C., Salas, A., Matsuyama, T., Sanchez-Torres, C., de la Torre, I., Miranda-Carus, M.E., Sanchez-Mateos, P., and Puig-Kroger, A. (2014). Macrophage uptake and accumulation of folates are polarization-dependent in vitro and in vivo and are regulated by activin A. J Leukoc Biol *95*, 797-808. 10.1189/jlb.0613345.

Sanchez-Martin, L., Estecha, A., Samaniego, R., Sanchez-Ramon, S., Vega, M.A., and Sanchez-Mateos, P. (2011). The chemokine CXCL12 regulates monocyte-macrophage differentiation and RUNX3 expression. Blood *117*, 88-97. 10.1182/blood-2009-12-258186.

Sanmamed, M.F., Rodriguez, I., Schalper, K.A., Onate, C., Azpilikueta, A., Rodriguez-Ruiz, M.E., Morales-Kastresana, A., Labiano, S., Perez-Gracia, J.L., Martin-Algarra, S., et al. (2015). Nivolumab and Urelumab Enhance Antitumor Activity of Human T Lymphocytes Engrafted in Rag2-/-IL2Rgammanull Immunodeficient Mice. Cancer Res *75*, 3466-3478. 10.1158/0008-5472.CAN-14-3510.

Schuchter, L.M. (2017). Adjuvant Melanoma Therapy - Head-Spinning Progress. N Engl J Med *377*, 1888-1890. 10.1056/NEJMe1711199.

Schwall, R.H., Robbins, K., Jardieu, P., Chang, L., Lai, C., and Terrell, T.G. (1993). Activin induces cell death in hepatocytes in vivo and in vitro. Hepatology *18*, 347-356. 10.1016/0270-9139(93)90018-i.

Seder, C.W., Hartojo, W., Lin, L., Silvers, A.L., Wang, Z., Thomas, D.G., Giordano, T.J., Chen, G., Chang, A.C., Orringer, M.B., and Beer, D.G. (2009a). INHBA overexpression promotes cell proliferation and may be epigenetically regulated in esophageal adenocarcinoma. J Thorac Oncol *4*, 455-462. 10.1097/JTO.0b013e31819c791a.

Seder, C.W., Hartojo, W., Lin, L., Silvers, A.L., Wang, Z., Thomas, D.G., Giordano, T.J., Chen, G., Chang, A.C., Orringer, M.B., and Beer, D.G. (2009b). Upregulated INHBA expression may promote cell proliferation and is associated with poor survival in lung adenocarcinoma. Neoplasia *11*, 388-396. 10.1593/neo.81582.

Si, T., Lu, Y., Li, F., Jiang, L., Pei, R., and Zhou, J.X. (2018). High expression of INHBA is an adverse prognostic factor for de novo acute myeloid leukemia. Leuk Lymphoma *59*, 114-120. 10.1080/10428194.2017.1324157.

Sica, A., and Bronte, V. (2007). Altered macrophage differentiation and immune dysfunction in tumor development. J Clin Invest *117*, 1155-1166. 10.1172/JCI31422.

Sica, A., and Mantovani, A. (2012). Macrophage plasticity and polarization: in vivo veritas. J Clin Invest *122*, 787-795. 10.1172/JCI59643.

Sierra-Filardi, E., Vega, M.A., Sanchez-Mateos, P., Corbi, A.L., and Puig-Kroger, A. (2010). Heme Oxygenase-1 expression in M-CSF-polarized M2 macrophages contributes to LPS-induced IL-10 release. Immunobiology *215*, 788-795. 10.1016/j.imbio.2010.05.020.

Smith, M.P., Sanchez-Laorden, B., O'Brien, K., Brunton, H., Ferguson, J., Young, H., Dhomen, N., Flaherty, K.T., Frederick, D.T., Cooper, Z.A., et al. (2014). The immune microenvironment confers resistance to MAPK pathway inhibitors through macrophage-derived TNFalpha. Cancer Discov *4*, 1214-1229. 10.1158/2159-8290.CD-13-1007.

Solinas, G., Germano, G., Mantovani, A., and Allavena, P. (2009). Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation. J Leukoc Biol *86*, 1065-1073. 10.1189/jlb.0609385.

Storr, S.J., Safuan, S., Mitra, A., Elliott, F., Walker, C., Vasko, M.J., Ho, B., Cook, M., Mohammed, R.A., Patel, P.M., et al. (2012). Objective assessment of blood and lymphatic vessel invasion and association with macrophage infiltration in cutaneous melanoma. Mod Pathol *25*, 493-504. 10.1038/modpathol.2011.182.

Sugama, S., Takenouchi, T., Kitani, H., Fujita, M., and Hashimoto, M. (2007). Activin as an anti-inflammatory cytokine produced by microglia. J Neuroimmunol *192*, 31-39. 10.1016/j.jneuroim.2007.08.016.

Tamminen, J.A., Yin, M., Ronty, M., Sutinen, E., Pasternack, A., Ritvos, O., Myllarniemi, M., and Koli, K. (2015). Overexpression of activin-A and -B in malignant mesothelioma - attenuated Smad3 signaling responses and ERK activation promote cell migration and invasive growth. Exp Cell Res *332*, 102-115. 10.1016/j.yexcr.2014.12.010.

Tanaka, T., Toujima, S., and Umesaki, N. (2004). Activin A inhibits growth-inhibitory signals by TGF-beta1 in differentiated human endometrial adenocarcinoma cells. Oncol Rep *11*, 875-879.

Tcyganov, E., Mastio, J., Chen, E., and Gabrilovich, D.I. (2018). Plasticity of myeloid-derived suppressor cells in cancer. Curr Opin Immunol *51*, 76-82. 10.1016/j.coi.2018.03.009.

Tripathi, M., Billet, S., and Bhowmick, N.A. (2012). Understanding the role of stromal fibroblasts in cancer progression. Cell Adh Migr *6*, 231-235. 10.4161/cam.20419.

Uribe-Querol, E., and Rosales, C. (2015). Neutrophils in Cancer: Two Sides of the Same Coin. J Immunol Res *2015*, 983698. 10.1155/2015/983698.

Vale, W., Rivier, J., Vaughan, J., McClintock, R., Corrigan, A., Woo, W., Karr, D., and Spiess, J. (1986). Purification and characterization of an FSH releasing protein from porcine ovarian follicular fluid. Nature *321*, 776-779. 10.1038/321776a0.

Vejda, S., Erlach, N., Peter, B., Drucker, C., Rossmanith, W., Pohl, J., Schulte-Hermann, R., and Grusch, M. (2003). Expression of activins C and E induces apoptosis in human and rat hepatoma cells. Carcinogenesis *24*, 1801-1809. 10.1093/carcin/bgg154.

Wakefield, L.M., and Roberts, A.B. (2002). TGF-beta signaling: positive and negative effects on tumorigenesis. Curr Opin Genet Dev *12*, 22-29. 10.1016/s0959-437x(01)00259-3.

Wan, Y.Y., and Flavell, R.A. (2007). 'Yin-Yang' functions of transforming growth factor-beta and T regulatory cells in immune regulation. Immunol Rev *220*, 199-213. 10.1111/j.1600-065X.2007.00565.x.

Wang, Y., Cui, X., Tai, G., Ge, J., Li, N., Chen, F., Yu, F., and Liu, Z. (2009). A critical role of activin A in maturation of mouse peritoneal macrophages in vitro and in vivo. Cell Mol Immunol *6*, 387-392. 10.1038/cmi.2009.50.

Welch, D.R., and Hurst, D.R. (2019). Defining the Hallmarks of Metastasis. Cancer Res *79*, 3011-3027. 10.1158/0008-5472.CAN-19-0458.

Wilms, H., Schwark, T., Brandenburg, L.O., Sievers, J., Dengler, R., Deuschl, G., and Lucius, R. (2010). Regulation of activin A synthesis in microglial cells: pathophysiological implications for bacterial meningitis. J Neurosci Res *88*, 16-23. 10.1002/jnr.22185.

Wunderlich, C.M., Ackermann, P.J., Ostermann, A.L., Adams-Quack, P., Vogt, M.C., Tran, M.L., Nikolajev, A., Waisman, A., Garbers, C., Theurich, S., et al. (2018). Obesity exacerbates colitis-associated cancer via IL-6-regulated macrophage polarisation and CCL-20/CCR-6-mediated lymphocyte recruitment. Nat Commun *9*, 1646. 10.1038/s41467-018-03773-0.

Wyckoff, J.B., Wang, Y., Lin, E.Y., Li, J.F., Goswami, S., Stanley, E.R., Segall, J.E., Pollard, J.W., and Condeelis, J. (2007). Direct visualization of macrophage-assisted tumor cell intravasation in mammary tumors. Cancer Res *67*, 2649-2656. 10.1158/0008-5472.CAN-06-1823.

Yamashita, N., Nakajima, T., Takahashi, H., Kaneoka, H., Mizushima, Y., and Sakane, T. (1993). Effects of activin A on IgE synthesis and cytokine production by human peripheral mononuclear cells. Clin Exp Immunol *94*, 214-219. 10.1111/j.1365-2249.1993.tb06003.x.

Yang, J.B., Zhao, Z.B., Liu, Q.Z., Hu, T.D., Long, J., Yan, K., and Lian, Z.X. (2018a). FoxO1 is a regulator of MHC-II expression and anti-tumor effect of tumor-associated macrophages. Oncogene *37*, 1192-1204. 10.1038/s41388-017-0048-4.

Yang, M., McKay, D., Pollard, J.W., and Lewis, C.E. (2018b). Diverse Functions of Macrophages in Different Tumor Microenvironments. Cancer Res *78*, 5492-5503. 10.1158/0008-5472.CAN-18-1367.

Yuen, G.J., Demissie, E., and Pillai, S. (2016). B lymphocytes and cancer: a love-hate relationship. Trends Cancer *2*, 747-757. 10.1016/j.trecan.2016.10.010.

Zauberman, A., Oren, M., and Zipori, D. (1997). Involvement of p21(WAF1/Cip1), CDK4 and Rb in activin A mediated signaling leading to hepatoma cell growth inhibition. Oncogene *15*, 1705-1711. 10.1038/sj.onc.1201348.

Zhang, J., Shen, K.W., Liu, G., Zhou, J., Shen, Q., Shen, Z.Z., and Shao, Z.M. (2003). Antigenic profiles of disseminated breast tumour cells and microenvironment in bone marrow. Eur J Surg Oncol *29*, 121-126. 10.1053/ejso.2002.1334.

Zhang, X.J., Li, Y., Tai, G.X., Xu, G.Y., Zhang, P.Y., Yang, Y., Lao, F.X., and Liu, Z.H. (2005). Effects of activin A on the activities of the mouse peritoneal macrophages. Cell Mol Immunol *2*, 63-67.

Zhou, J., Tai, G., Liu, H., Ge, J., Feng, Y., Chen, F., Yu, F., and Liu, Z. (2009). Activin A down-regulates the phagocytosis of lipopolysaccharide-activated mouse peritoneal macrophages in vitro and in vivo. Cell Immunol *255*, 69-75. 10.1016/j.cellimm.2008.11.001.

ANEXO

Publicaciones relacionadas

La expresión de CCL20 por macrófagos asociados a tumor predice la progresión del melanoma cutáneo primario humano

El eje de quimiocinas CCR6/CCL20 está involucrado en la progresión del cáncer en una variedad de tumores. Aquí, mostramos que CCR6 es expresado por células de melanoma. El ligando de CCR6, CCL20, induce la migración y la proliferación *in vitro* y mejora el crecimiento tumoral y la metástasis *in vivo*. El análisis con confocal de tejidos de melanoma mostró que CCR6 se expresa en células tumorales, mientras que CCL20 se expresa primordialmente en células no tumorales en el estroma de ciertos tumores. El CCL20 estromal, pero no el CCR6 tumoral, predijo una mala supervivencia en una cohorte de 40 pacientes con melanoma primario. Los macrófagos asociados a tumores (TAM), independientemente de su perfil de polarización M1/M2, se identificaron como la principal fuente de CCL20 en melanomas primarios que desarrollaron metástasis. Además de CCL20, los TAM expresaron citoquinas pro-tumorales TNF y VEGF-A, lo que sugiere que la progresión del melanoma está respaldada por macrófagos con un estado de activación diferencial. Nuestros datos destacan la interacción sinérgica entre las células tumorales de melanoma y los macrófagos pro-metastásicos a través de un bucle paracrino CCR6/CCL20. Los niveles estromales de CCL20 en melanomas primarios pueden ser un marcador clínicamente útil para evaluar el riesgo del paciente, tomar decisiones de tratamiento y planificar o analizar ensayos clínicos.

Samaniego R, Gutiérrez-González A, **Gutiérrez-Seijo A**, Sánchez-Gregorio S, García-Giménez J, Mercader E, Márquez-Rodas I, Avilés JA, Relloso M, Sánchez-Mateos P. CCL20 Expression by Tumor-Associated Macrophages Predicts Progression of Human Primary Cutaneous Melanoma. Cancer Immunol Res. 2018 Mar;6(3):267-275. doi: 10.1158/2326-6066.CIR-17-0198. Epub 2018 Jan 23. PMID: 29362221.

Research Article

Cancer Immunology Research

CCL20 Expression by Tumor-Associated Macrophages Predicts Progression of Human Primary Cutaneous Melanoma

Rafael Samaniego[1], Alejandra Gutiérrez-González[2], Alba Gutiérrez-Seijo[1,2], Sandra Sánchez-Gregorio[1,2], Jorge García-Giménez[1,2], Enrique Mercader[3], Iván Márquez-Rodas[4], José Antonio Avilés[5], Miguel Relloso[6], and Paloma Sánchez-Mateos[2,7]

Abstract

The chemokine axis CCR6/CCL20 is involved in cancer progression in a variety of tumors. Here, we show that CCR6 is expressed by melanoma cells. The CCR6 ligand, CCL20, induces migration and proliferation *in vitro,* and enhances tumor growth and metastasis *in vivo*. Confocal analysis of melanoma tissues showed that CCR6 is expressed by tumor cells, whereas CCL20 is preferentially expressed by nontumoral cells in the stroma of certain tumors. Stromal CCL20, but not tumoral CCR6, predicted poor survival in a cohort of 40 primary melanoma patients. Tumor-associated macrophages (TAM), independently of their M1/M2 polarization profile, were identified as the main source of CCL20 in primary melanomas that developed metastasis. In addition to CCL20, TAMs expressed TNF and VEGF-A protumoral cytokines, suggesting that melanoma progression is supported by macrophages with a differential activation state. Our data highlight the synergistic interaction between melanoma tumor cells and prometastatic macrophages through a CCR6/CCL20 paracrine loop. Stromal levels of CCL20 in primary melanomas may be a clinically useful marker for assessing patient risk, making treatment decisions, and planning or analyzing clinical trials. *Cancer Immunol Res; 6(3); 267–75. ©2018 AACR.*

Introduction

Chemokines and their receptors are involved in multiple aspects of cancer progression. In addition to their chemotactic role guiding the metastatic process, chemokines can provide survival signals to cancer cells that promote tumor growth in otherwise nonsupportive microenvironments. Chemokines can also indirectly modulate tumor growth through their effect on nontumor cells in the stroma by inducing release of growth and angiogenic factors and by directing the trafficking of both antitumor and protumor leukocytes. Chemokines also participate in the response to cancer therapies and are targets for immunotherapy and chemokine-targeted therapy (1).

Cutaneous melanoma is an aggressive skin tumor in which chemokines and their receptors regulate progression (2). Originally described as autocrine factors for melanoma growth, the chemokines CXCL8 (IL8) and CXCL1-2 (GRO1-2), as well as their respective receptors CXCR1 and CXCR2, regulate melanoma tumor progression by affecting tumor growth, angiogenesis, and metastasis (3). CXCR4 expression renders melanoma cells responsive to CXCL12 and drives local invasion and metastasis (4, 5), and its expression in primary melanoma patients is associated with a higher metastatic risk and mortality rate (6). Expression of certain chemokine receptors by melanoma cells is associated with selective organ metastases with localization dependent on expression of particular ligands: CCR7 is associated with lymph node, which is rich in CCL19 and CCL21; CCR9 is associated with small intestine, which is rich in CCL25; and CCR10 is associated with metastasis in the skin, which is rich in CCL27 (7, 8).

Overexpression of CCR6 by cancer cells and aberrant signaling by its ligand CCL20 has been documented in many cancer types including colorectal, hepatocellular, pancreatic, ovarian, nasopharyngeal, and breast cancer (9–12). The purpose of this study was to characterize CCR6/CCL20 signaling and expression in human melanoma. We found that CCR6 is expressed by melanoma cells, whereas CCL20 is expressed by stromal cells in certain primary cutaneous melanomas. CCL20 stromal expression was associated with metastatic progression and patient survival. The stromal source of CCL20 in primary melanomas that developed metastasis was identified as protumoral TAMs. These cells coexpress TNF and VEGF, independently of their M1 or M2 profile.

[1]Unidad de Microscopía Confocal, Instituto de Investigación Sanitaria Gregorio Marañón (IiSGM), Madrid, Spain. [2]Laboratorio de Inmuno-oncología, Instituto de Investigación Sanitaria Gregorio Marañón (IiSGM), Madrid, Spain. [3]Servicio de Cirugía General, Hospital General Universitario Gregorio Marañón (HGUGM), Madrid, Spain. [4]Servicio de Oncología, Hospital General Universitario Gregorio Marañón (HGUGM), Madrid, Spain. [5]Servicio de Dermatología, Hospital General Universitario Gregorio Marañón (HGUGM), Madrid, Spain. [6]Grupo de Inmunofisiología, Instituto de Investigación Sanitaria Gregorio Marañón (IiSGM), Madrid, Spain. [7]Departamento de Inmunología, Facultad de Medicina, Universidad Complutense de Madrid, Madrid, Spain.

Note: Supplementary data for this article are available at Cancer Immunology Research Online (http://cancerimmunolres.aacrjournals.org/).

Corresponding Authors: Rafael Samaniego, IISGM, c/ Maiquez, 9, Madrid 28009, Spain. Phone: 915-868-739; Fax: 915-868-018; E-mail: confocal@hggm.es; or Paloma Sánchez-Mateos, Laboratorio de Inmunooncología, Instituto de Investigación Sanitaria Gregorio Marañón, c/ Dr. Esquerdo, 46, Madrid 28007, Spain. E-mail: paloma.sanchezmateos@salud.madrid.org

doi: 10.1158/2326-6066.CIR-17-0198

Materials and Methods

Cells and antibodies

Human melanoma cells and associated fibroblasts were isolated from biopsied metastatic lesions, as previously described (13). TAMs and tumor-infiltrating lymphocytes (TIL) were purified by magnetic cell sorting using CD14 and CD3 microbeads (Miltenyi-Biotech) from human-homogenized melanomas (Miltenyi-Biotech), respectively. Monocytes were isolated with CD14 microbeads from healthy donors or melanoma patients' peripheral blood. For *in vitro* generation of macrophages and dendritic cells (DC), monocytes were incubated with GM-CSF (M1), M-CSF (M2), or GM-CSF/IL4 (iDC) for 7 days renewing cytokines every 3 days, as previously described (14). All cells, melanoma cell lines Mewo, BLM, A375, MV3, Skmel103, and Skmel147 (15); MEL-F melanocytes, Caco-2, NCI-H929, and Hek-293 (from ATCC) were grown in RPMI-1640 medium (Gibco) supplemented with 10% FCS (Harlan Sera-lab). Cells lines were mycoplasma-free and used before fifth passage from thawing. Cell lines were not reauthenticated during the last year, but checked for melanoma markers (HMB45, NG2, S100). Primary antibodies are specified in Supplementary Table S1.

In vitro measurements

For coculture assays, 5×10^4 melanoma cells were first cultured for 3 days in 12-well plates, and then 10^6 macrophages were added to each well. After 72 hours, coculture supernatants were collected for CCL20 assessment (DouSet-DY360; R&D Systems) and cells processed for quantitative real-time PCR (qPCR) analyses, separating macrophages from melanoma cells with CD14 microbeads. Oligonucleotides (Supplementary Table S1) were designed according to the Roche software for qPCR. Total RNA was extracted (RNeasy-kit; Qiagen) and retrotranscribed cDNA quantified using the Universal Human Probe Roche library (Roche-Diagnostics). Assays were made in triplicate and normalized to *TBP* and/or *GAPDH* expression (ΔΔCT method). Western blot and flow cytometry analyses were performed as previously described (14, 15).

Migration and proliferation assays

For chemotactic assays, cells were suspended in RPMI-1640 containing 0.1% FCS and 5×10^4 cells added on top of 8 μm pore transwell chambers (Costar). Then, cells were allowed to migrate for 4 hours at 37°C toward 0.1% FCS RPMI-1640 containing recombinant human chemokines (R&D Systems; PreproTech). Nonmigrated cells were removed from the upper part of the membranes with cotton swabs, and migrated cells attached to the bottom part were quantified. For wound-closure assays, 5×10^4 cells suspended in 1% FCS RPMI-1640 were added within wells of 1-mm insets (IBIDI) placed on 2 μg/mL collagen I (Nutragen) coated 24-well plastic plates and allowed to attach overnight. Then, insets were removed, plate wells were washed twice, and attached cells were allowed to migrate for 4 hours in 0.1% FCS RPMI-1640 ± rhCCL20 (R&D Systems). For quantification in both assays, cells were fixed with 4% formaldehyde, stained with propidium iodide (Sigma), and imaged at five random fields with a 10x objective with a fluorescence camera (sCMOS Orca-Flash-4.0/LT). Number of migrated cells and wound-closure distances were calculated using the FIJI software (National Institute Health, US). For proliferation assays, 10^5 cells were suspended in 1% BSA RPMI-1640 ± chemokines (R&D Systems), seeded on collagen-coated coverslips, and incubated for 24 hours at 37°C, adding bromodeuxyuridine (BrdUrd; Roche) 4 hours before fixation. Cells were then stained for BrdUrd and percentages of replicated nuclei estimated. This assay was used to study the effect of CCR6 downregulation in BLM cells using magnetofection (Biosciences) with siRNA (CKR-6/sc-35064; Santa Cruz Biotechnology) or blocked with anti-hCCR6 (clone 53103).

Melanoma xenograft models

NSG mice (The Jackson Laboratory) were maintained under specific pathogen-free conditions. At age 4 to 6 weeks, male mice were subcutaneously inoculated with 10^6 melanoma cells suspended in PBS ± 200 ng/mL rhCCL20 (R&D Systems; PreproTech). Mice were then subcutaneously injected with PBS ± 500 ng rhCCL20 every 3 days until tumors reached approximately 1 cm width, when they were resected, weighed, and frozen for further histologic analyses. For *in vivo* CCR6 blockade assays, mice were inoculated with 10^6 tumor cells (TCs) in PBS containing 10 μg/mL blocking anti-hCCR6 or IgG2b mouse antibodies, ±200 ng/mL rhCCL20 (added 30 minutes afterwards). Mice were then intravenously injected with 100 μg antibodies, ±500 ng rhCCL20 (supplied subcutaneously 2 hours later) every 3 days. In BLM tumor-bearing mice, spontaneous metastases were examined 10 days after resection of primary tumors, when lungs were extracted and fixed with Bouins fluid to quantify superficial metastases. These procedures were approved by the IiSGM animal care/use and Comunidad de Madrid committees (PROEX-14214).

Fluorescence confocal microscopy

Melanoma tissues were obtained from patients with primary and metastatic lesions who were undergoing surgical treatment, following the medical-ethics committee procedures of HGUGM. Fifty-seven cryopreserved melanoma samples were screened: 41 primary tumors and 16 metastases. Cryosections (5 μm) were fixed with cold acetone for 15 minutes, stained with different antibodies, and analyzed by confocal microscopy. For survival analyses, primary melanomas were triple-stained with CCR6 (clone 53103.111), CCL20 (ab9829), and Hmb45 antibodies. Then, several 20x fields (3–5 per sample) were analyzed at regions of interest (10–15 per field) identified along tumor ($Hmb45^+$) and stromal zones, to measure tumor-CCR6 and stromal-CCL20 mean fluorescence intensities (MFIs), respectively. For *in vivo* TAM phenotyping, CD163-, CD163/CD11c-, CD11c/CD209-, or CD115/CD15-stained cells were segmented, and MFI of distinct markers was quantified at matched single cells. Similarly, for *in vivo* proliferation quantification, DAPI-stained nuclei were segmented and Ki67 MFI assessed. All quantifications were performed in a blinded way. Imaging was performed using the glycerol ACS APO 20x NA0.60 and oil ACS APO 40x NA1.30 immersion objectives of a confocal fluorescence microscope (SPE, Leica-Microsystems), and FIJI software was used for image quantification in all cases.

Prognostic significance analysis

To evaluate the prognostic significance of tumor-CCR6 and stromal-CCL20 expression in melanoma progression and survival, we used a cohort of 40 frozen human skin primary melanomas, obtained between the years 1999 and 2013, with known clinicopathologic features, including age, gender, tumor thickness (Breslow), ulceration, histologic subtype, location, American

Joint Committee on Cancer stage, metastasis occurrence, and survival within the next 5 years to primary tumor resection. MFI values corresponding to two variables, stromal-CCL20 and tumor-CCR6, were classified into high or low levels using the median as cutoff points. Censured Kaplan–Meier curves were used to analyze the correlation with patient disease-free and overall survival; and the Cox-regression method (univariate and multivariate) was used to identify independent prognostic variables. The Mann–Whitney *t* test was used to evaluate the association with clinicopathologic features.

Statistical analyses

The Student *t* test, Mann–Whitney, Wilcoxon paired test, Spearman correlation, Cox-regression, and Log-rank analyses have been used in this study, as indicated. $P < 0.05$ was considered statistically significant.

Results

CCR6/CCL20 functional expression by melanoma cells

CCR6 expression by patient-derived melanoma cells and cell lines was investigated. Melanoma tumors are complex tissues infiltrated by leukocytes which may express CCR6. Therefore, for PCR and other analyses, leukocytes were removed from patient samples with the use of anti–CD45-coated magnetic beads. All cell lines studied and patient-derived TCs expressed CCR6 mRNA, compared with other cells known to express or not CCR6 (Fig. 1A). CCR6 protein expression was confirmed by Western blot, flow cytometry, and immunofluorescence. A specific 50-kDa band was detected in all melanocytic cells studied, with a variable but measurable CCR6 surface staining and a larger intracellular pool detected by flow cytometry of intact or permeabilized cells, respectively (Fig. 1B and C; Supplementary Fig. S1A–S1D).

To explore CCR6/CCL20 signaling, we performed cell proliferation and migration assays (Fig. 1D–F). CCL20 induced migration of BLM cells in wound-closure chemokinetic assays and in chemotactic assays, with a bell shape dose-response curve, whereas A375 cells responded to a broad range of CCL20 concentrations. CCL20 enhanced proliferation of BLM and A375 cells similarly to CXCL12 or CXCL8, which signal through other chemokine receptors, and showed no additional effects when the three chemokines were used together (CCL20, CXCL12, and CXCL8). To confirm specificity of CCR6/CCL20 signaling, we used either siRNA to silence CCR6 expression or mAb to CCR6 to block the receptor, showing abrogation of exogenous CCL20 effects but not of CXCL12 (Fig. 1G and H). These results indicated that melanoma cells express functional CCR6, which may contribute to proliferation and migration in response to exogenous CCL20.

CCL20 enhances tumor growth and metastasis in melanoma mouse models

In order to determine the effect of exogenous CCL20 on tumor growth, we developed xenografts with BLM, A375, and Skmel147 human melanoma cells, which were injected subcutaneously into NSG mice. Mice were then treated every 3 days with hCCL20 or

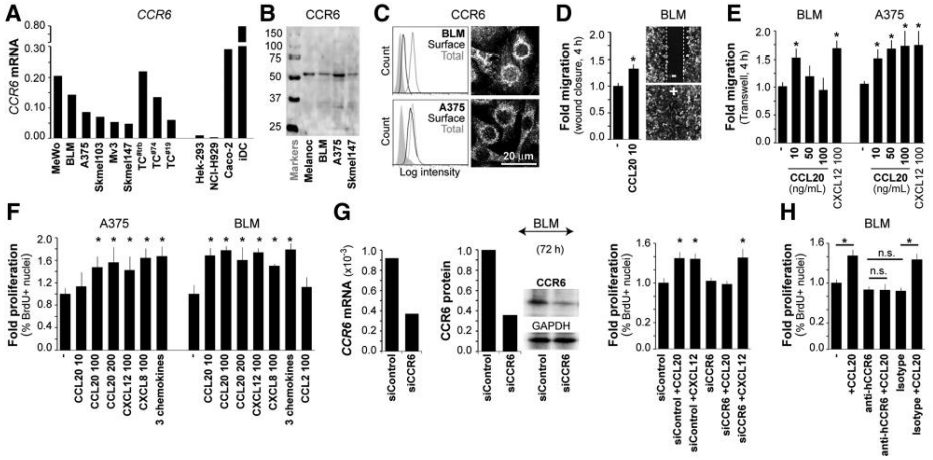


Figure 1.

Melanoma cells express CCR6 and respond to exogenous hCCL20. **A,** CCR6 mRNA expression (relative to *TBP*) in melanoma cells compared with other cell types (Hek-293 and NCI-H929 as negative controls; Caco-2 and iDC as positive controls). **B** and **C,** CCR6 protein expression in human melanocytes and melanoma cell lines as assessed by Western blot (**B**); flow cytometry (isotype, shaded; surface, black-line; total/permeabilized, gray line) and permeabilized cells immunofluorescence (**C**). **D** and **E,** Migratory response to exogenous chemokines (ng/mL), as assessed by wound-closure (**D**) and transwell (**E**) assays. **F,** Proliferative response to exogenous chemokines (ng/mL), as indicated. **G** and **H,** Proliferation assays achieved with BLM cells downregulated for CCR6 with siRNA (72 hours, **G**), or blocked with 10 μg/mL anti-hCCR6 antibody (**H**). CCR6 silencing was confirmed at both mRNA (relative to *TBP*/*GAPDH* average) and protein (normalized to GAPDH) levels (**G**). **D** to **H,** Data are given as the mean ± SD from two to three independent experiments performed in triplicate. Significant differences respective to untreated controls are shown (*, $P < 0.05$; *t* Student). Scale bar, as indicated.

vehicle; reaching concentrations >200 pg of hCCL20 per gram of tumor tissue after 0.5 hours of injection (Fig. 2A; Supplementary Fig. S1E–S1G). Exogenous hCCL20 increased tumor growth by 2-fold in BLM ($P = 0.001$) and A375 ($P = 0.011$) models. Significant differences were found with Skmel147 cells ($P = 0.016$). Both BLM and A375 tumors showed more proliferating Ki67$^+$ cells in hCCL20-treated mice (Fig. 2B). We also analyzed vascularization and leukocyte infiltration in order to account for indirect host-mediated effects. No differences were found in stromal components between hCCL20- and vehicle-treated mice (Fig. 2C and D). CCR6 expression was limited to malignant human cells, whereas key host cells, such as F4/80$^+$ TAMs, lacked CCR6 expression (Supplementary Fig. S1H). A blocking mAb to human CCR6 abrogated the effect on tumor growth of exogenous hCCL20 (Fig. 2E). In the absence of hCCL20 treatment, no differences were observed between xenografts treated with anti-CCR6 or isotype control Ab, precluding any role of endogenous CCL20. Next, to analyze the impact of hCCL20 treatment in the ability of BLM cells to metastasize to distant organs, we used a model of spontaneous metastasis. Lung weight and number of superficial metastases were significantly higher in mice treated with hCCL20 than those treated with vehicle (Fig. 2F). These results indicated that hCCL20 administration enhanced both local and metastatic tumor growth in xenograft models.

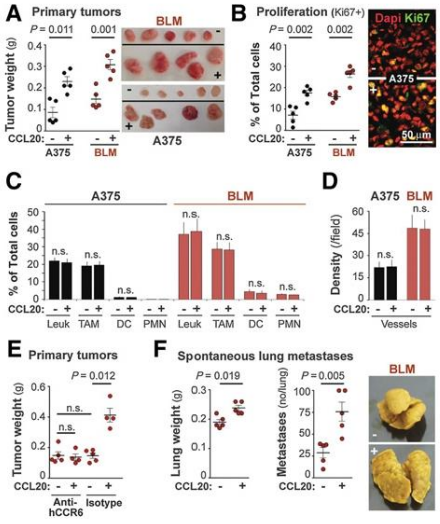


Figure 2.

Exogenous hCCL20 induces melanoma tumor progression *in vivo*. **A,** Human melanoma xenografts (BLM, A375) developed in NSG mice subcutaneously injected with hCCL20 (+) or PBS (−) every 3 days ($n = 5$ mice/condition). Photographs of resected tumors and their weight values are shown. **B,** Percentages of proliferating cells (Ki67$^+$ nuclei) relative to total cells (DAPI$^+$ nuclei). Right, Ki67 (green) and DAPI (red) staining of A375 tumors from mice treated (+) or untreated (−) with hCCL20. Scale bar, 50 µm. **C,** Percentages of immune cells infiltrating tumors relative to total cells, including leukocytes (Cd45$^+$), TAMs (F4/80$^+$), DC (F4/80$^-$ Cd11c$^+$), and neutrophils (PMN, F4/80$^-$ Ly6g$^+$). **D,** Intratumoral Cd31$^+$ vessel densities. **E,** *In vivo* blockade of hCCR6 during melanoma xenograft development in mice treated (+) or untreated (−) with exogenous hCCL20 ($n = 4$–5 mice/condition). Tumor weights are shown. **F,** Lung metastases 10 days after BLM primary tumors resection. Weight values, number of superficial metastases ("100", when uncountable), and lungs fixed with Bouins solution are shown. *P* values are shown (Mann–Whitney test, n.s., nonsignificant).

CCR6/CCL20 expression in primary cutaneous melanoma and metastasis

We next examined whether CCR6/CCL20 expression might have translational value by analyzing correlation with clinicopathologic factors and patient survival. We screened a collection of 40 primary cutaneous melanomas with a median patient follow-up of 67 months (range, 5–187 months) and 14 metastases (Supplementary Table S2). Frozen tissue sections were simultaneously triple-labeled for the melanosome protein Hmb45, CCR6, and CCL20 (Fig. 3A). The majority of Hmb45$^+$ cells coexpressed CCR6 in all primary and metastatic lesions. By contrast, CCL20 was expressed in the stromal compartment. Therefore, to understand the prognostic value of CCR6/CCL20 expression in primary cutaneous melanoma, we quantified the MFI of CCR6 staining along Hmb45$^+$ tumor nests, whereas CCL20 expression was evaluated at Hmb45$^-$ stromal bundles (Fig. 3B and C). MFI data of tumor-CCR6 and stromal-CCL20 expression were compared with clinicopathologic factors: higher expression of stromal-CCL20 correlated with unfavorable prognostic factors for cutaneous melanomas such as tumor thickness >2 mm ($P = 0.023$) and stage II–III ($P = 0.002$), whereas nonsignificant correlation with progression indicators was found for tumoral-CCR6 levels (Supplementary Table S3). Next, we used the Kaplan–Meier method to analyze whether tumor-CCR6 or stromal-CCL20 expression correlated with disease-free survival or overall survival of this group of 40 patients (Fig. 3D). We classified patients based on their respective tumor-CCR6 or stromal-CCL20 expression levels as "high" or "low," using the median MFI value as cutoff point. High stromal-CCL20 staining correlated with shorter disease-free and overall survival (log-rank test, both $P < 0.001$), but no correlation was found between tumor-CCR6 staining and disease-free survival ($P = 0.693$). Finally, to determine whether stromal-CCL20 expression was an independent prognostic factor, we performed a multivariate regression analysis including gender, age, Breslow, and stage parameters (Table 1). This analysis showed that stromal-CCL20 expression level was an independent prognostic factor of disease-free survival [HR, 1.5; 95% confidence interval (CI), 1.25–1.76; $P < 0.001$] and overall survival (HR, 1.7; 95% CI, 1.2–2.5; $P = 0.004$) in this cohort. These findings highlighted the role of CCL20 expression by the stroma in predicting clinical behavior of primary cutaneous melanoma patients.

TAMs are the major stromal source of CCL20 in human melanoma tissues

To identify the CCL20 source in melanoma tumors, we isolated malignant cells and infiltrating leukocytes as macrophages and T cells (ref. 13; Fig. 4A; Supplementary Fig. S2A). To isolate TAMs, we used CD14 that was expressed by macrophages and correlated with CD163 (Supplementary Fig. S2B). TAMs showed higher expression of CCL20 mRNA than CD3$^+$ TILs, malignant cells, cancer-associated fibroblasts (CAFs), or peripheral blood monocytes from the same patients. CCL20

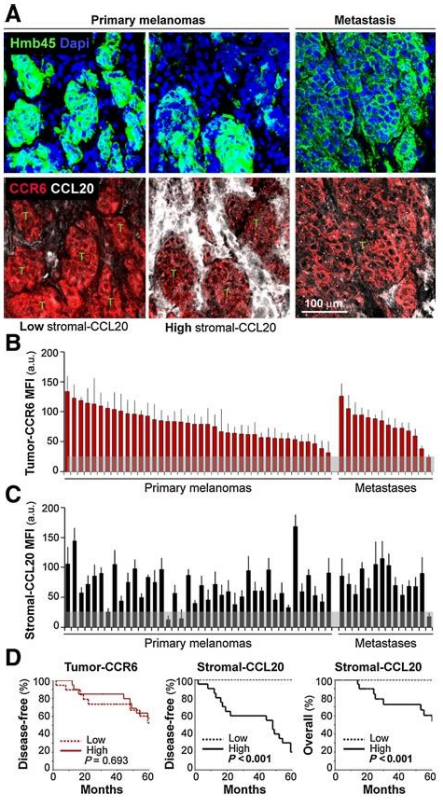


Figure 3.

CCR6/CCL20 expression in human melanoma and correlation with patient survival. **A,** Human melanoma samples stained for Hmb45 (green), CCR6 (red), and CCL20 (white). Primary tumors with different stromal-CCL20 expression ("low" and "high") are shown. "T," tumor nest. Scale bar, 100 μm. **B** and **C,** Quantification of tumor-CCR6 (**B**) and stromal-CCL20 (**C**) expression. MFI ± SD (in arbitrary units, a.u.) are shown. Samples (40 primary tumors and 14 metastases) follow the same order in both histograms. **D,** Five-year Kaplan–Meier curves for tumor-CCR6 (disease-free survival) and stromal-CCL20 (disease-free and overall survival). The median values of the 40 primary melanomas were used to classify as "low" or "high" expressing samples (79 a.u. for tumor-CCR6; 72 a.u. for stromal-CCL20). *P* values are shown (log-rank).

protein secretion measured in the culture supernatant of TAMs, CAFs, and melanoma cells showed that TAMs were actively secreting large amounts of this chemokine (Fig. 4B). As expected, tissue supernatant from a mechanically disaggregated advanced primary melanoma also contained CCL20 (Fig. 4C). Therefore, we focused our interest in TAMs as the predominant source of CCL20 in melanoma tissues. However, neither proinflammatory (GM-CSF) M1 nor ant-inflammatory

Table 1. Univariate and multivariate Cox-regression analysis for disease-free survival

Disease-free survival (Cox regression)	Univariate	Multivariate	
	P	HR (95% CI)	*P*
Gender (F vs. M)	0.070	5.307 (1.26–22.2)	0.023
Age (years)	0.919	1.022 (0.99–1.05)	0.123
Location (H/L vs. T)	0.547		
Subtype (nod vs. others)	0.246		
Ulceration (yes vs. no)	0.267		
Breslow (mm)	<0.001	1.896 (1.33–2.69)	<0.001
Stage (I vs. II–III)	<0.001	1.433 (0.69–2.95)	0.330
T-CCR6 (MFI, each 10 a.u.)	0.490		
S-CCL20 (MFI, each 10 a.u.)	<0.001	1.487 (1.25–1.76)	<0.001

(M-CSF) M2 *in vitro*–differentiated macrophages secreted significant amounts of the chemokine (Fig. 4C), suggesting that the CCL20 high ($CCL20^{hi}$) phenotype might be a distinctive TAM characteristic. Tissue supernatant from primary melanoma induced CCL20 secretion and mRNA upregulation by both M1 and M2 macrophages (Fig. 4C and D). Moreover, high CCL20 concentrations were secreted during coculture of M1 (∼100 fold) or M2 (∼50 fold) macrophages with BLM and A375 cells, but not with Skmel103 cells, compared with each corresponding cell type cultured alone (Fig. 4C). M1 macrophages separated from melanoma cells after coculture showed higher CCL20 mRNA expression than M2 macrophages or melanoma cells (Fig. 4D). These results indicated that primary tumor-derived factors or interactions with melanoma cells induced the $CCL20^{hi}$ secretory phenotype in both M1 and M2 macrophages.

Next, we analyzed macrophage CCL20 expression in tissues by multicolor confocal microscopy, including control skin, nevi, primary melanomas (grouped as nonmetastatic and metastatic primary tumors), and distant melanoma metastases (Fig. 4E and F). To quantify CCL20 at the single-cell level, we used CD163 to gate tissue macrophages because it is expressed by most macrophages (16). A subpopulation of TAMs (∼50%) showing $CCL20^{hi}$ (stated as MFI >75 a.u.) were detected in primary cutaneous melanomas from patients that developed metastasis during follow-up (referred from here as "metastatic primary melanomas"), whereas most TAMs from nonmetastatic primary tumors showed $CCL20^{low}$ (<75 a.u.), similar to skin and nevus macrophages. In secondary metastatic melanoma, the $CCL20^{hi}$ TAM subpopulation was observed in some samples but not in others. CCL20 MFI amounts correlated in single cells and stromal regions from primary tumors ($R = 0.727$), whereas no significant correlation was obtained between TAM density and stromal-CCL20 (Supplementary Fig. S2C and S2D). A minority of $CCL20^{hi}$ cells were $CD163^-$ and $CD11c^+$ (Fig. 4G). We used DC markers (CD141 for DC1 subset and CD1c for DC2 subset) to characterize these cells as $CD11c^+CD1c^+$ classical DC2 (Fig. 4G and H; Supplementary Fig. S2E). In addition to being expressed by DC, CD11c was mostly expressed by a subset of $CD14^+$ and $CD115^+$ macrophages. These TAMs displayed lower CD163 expression and fell into two subsets: $CD11c^{hi}CD163^{low}$ and $CD11c^{low}CD163^{hi}$ (Fig. 4H). $CCL20^{hi}$ expression was detected in all these myeloid subsets in primary tumors from patients that developed metastasis but not in tumors from nonmetastatic patients (Fig. 4G). In contrast, CCL20 was only barely detectable in polymorphonuclear cells (PMNs), identified as $CD15^+CD115^-$ minor subset (Fig. 4I).

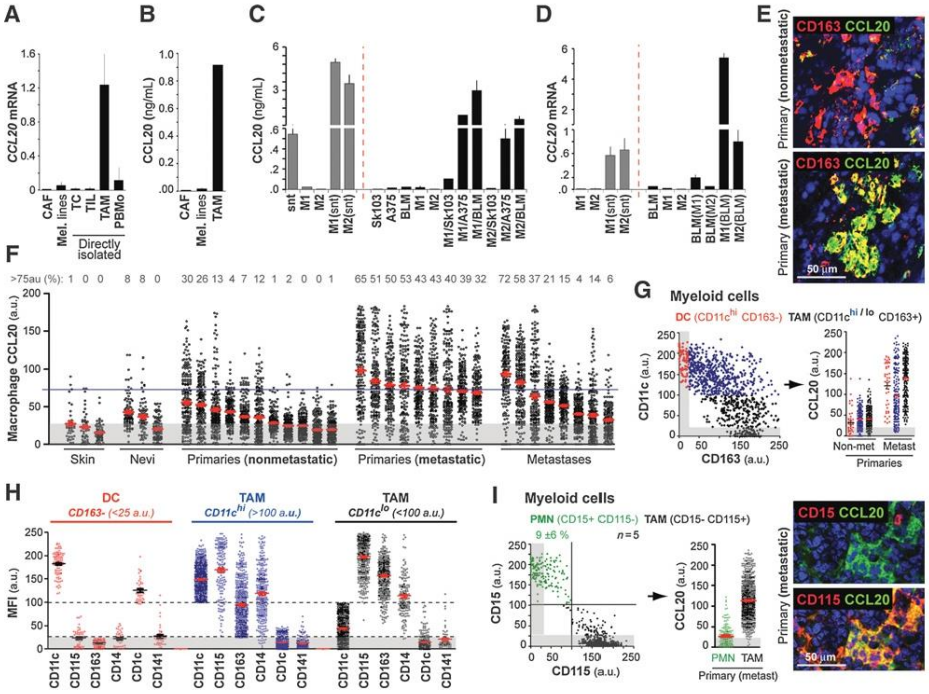


Figure 4.

TAMs are the major source of CCL20 in metastatic primary melanomas. **A,** Relative expression of CCL20 mRNA in cultured melanoma cell lines ($n = 6$, as in Fig. 1A) and CAF ($n = 3$ patients); and in freshly isolated TCs ($n = 3$), TILs CD3$^+$ ($n = 3$), TAMs CD14$^+$ ($n = 5$), and peripheral blood monocytes CD14$^+$ from melanoma patients (PBMo, $n = 3$). **B,** CCL20 detected in supernatants of CAF ($n = 3$ patients), melanoma cell lines ($n = 6$, as in Fig. 1A), and isolated TAMs CD14$^+$ ($n = 3$) after 24-hour incubation. **C,** Left, CCL20 secreted by M1 or M2 macrophages conditioned with an advanced primary melanoma tissue-supernatant (snt) for 48 hours ($n = 3$). Right, CCL20 detected in culture-supernatants of *in vitro*–differentiated macrophages (M1 or M2; $n = 3$) and TCs (Skmel103, A375, or BLM), alone or cocultured for 72 hours, as indicated. **D,** CCL20 mRNA expression in mutually conditioned, separated macrophages, and BLM cells ($n = 3$), or in tissue-supernatant conditioned macrophages. **A–D,** Data are given as the mean ± SD. **E,** Representative metastatic and nonmetastatic primary tumors stained for CD163 (red), CCL20 (green), and DAPI (blue). **F,** Dot plot showing CCL20 MFI values of single CD163$^+$ macrophages from control skin, nevus, primary tumors (nonmetastatic and metastatic), and metastasis tissues. Mean ± standard error, and the percentage of TAMs expressing CCL20^{high} (arbitrarily assigned to MFI > 75 a.u.), are shown for each sample. **G,** CCL20 MFI quantified in single-cell myeloid subpopulations of DC (red dots, CD11c$^+$ CD163$^-$) and TAMs (blue, CD11c^{hi} CD163$^+$; black, CD11c^{low} CD163$^+$) from metastatic and nonmetastatic primary tumors ($n = 3$/group). **H,** DC (CD1c, CD141) and macrophage (CD115, CD14, CD163) markers MFI quantified in DC and TAM subsets ($n = 5$), as in **G**. **I,** CCL20 MFI quantified in single-cell myeloid subpopulations of PMN (green, CD15$^+$ CD115$^-$) and TAMs (black, CD15$^-$ CD115$^+$) from 5 metastatic primary tumors. Illustrative images are shown, as indicated. Scale bars, 50 µm.

Protumoral TAMs secrete CCL20, regardless of polarization M1/M2 state

Because CD11c/CD163 expression identified two TAM subsets and CD11c expression in macrophages may indicate skewing toward M1 polarization (17–19), we analyzed in more detail M1/M2 polarization markers (17, 20, 21). Single-cell quantitative analysis of melanoma tissues showed a positive correlation between CD11c and pSTAT1 M1 markers and between CD163L1 and CD209 M2 markers; whereas M1 (CD11c and pSTAT1) and M2 (CD163L1 and CD209) markers correlated negatively between them (Supplementary Fig. S3A). The best negative correlation was obtained between CD11c and CD209 ($R = -0.75$; $P < 0.001$) allowing identification of the following subsets: CD11c^{hi}CD209$^{low/-}$ M1-like TAMs; CD11c$^{low/-}$CD209^{hi} M2-like TAMs; and a mixed M1/M2 population expressing both markers equally (Fig. 5A and B). Diverse TAM subsets displaying M1, M2, or M1/M2 mixed profiles were found in ten primary melanomas, with no significant differences in subset distribution with regard to subsequent nonmetastatic or metastatic patient evolution (Supplementary Fig. S3B). CCL20^{hi} expression was detected independently of M1/M2 polarization state in most TAMs from the metastatic group (Fig. 5B), compared with low CCL20 expression by most TAMs from nonmetastatic melanomas (Figs. 4F and 5A). Altogether, these results suggested that CCL20 expression by TAMs in primary melanoma tumors may be the result of a metastatic activation state. TAM CCL20 expression might predict

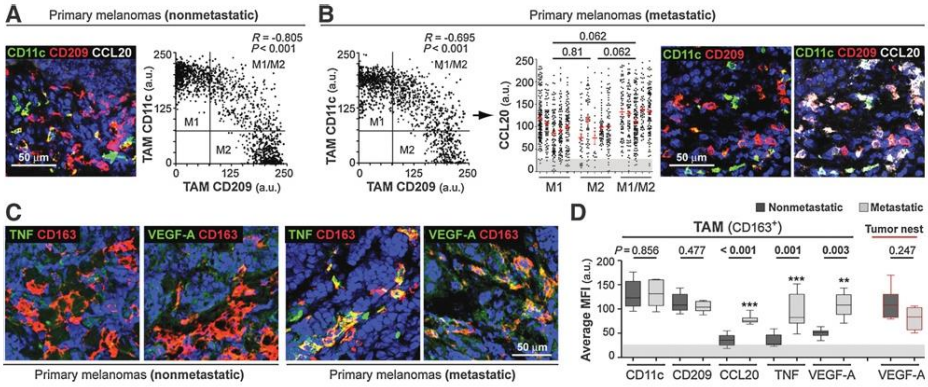


Figure 5.

CCL20 is expressed by TAMs displaying a protumoral profile, regardless of their polarization state. **A** and **B,** Nonmetastatic (**A**) and metastatic (**B**) primary tumors stained for CD11c (green), CD209 (red), CCL20 (white), and DAPI (blue). Dot plots of TAM subpopulations categorized as $CD11c^{hi}$ $CD209^{low}$ (M1-like), $CD11c^{low}$ $CD209^{hi}$ (M2-like), and mixed phenotype (M1/M2), using 75 a.u. as arbitrary cutoff MFI point ($n = 5$/group; R, Spearman's coefficients). **B,** TAM CCL20 MFI values are shown for metastatic primary tissues (Wilcoxon paired test). **C,** Representative nonmetastatic and metastatic primary tumors stained for TNF (green), VEGF-A (green), CD163 (red), and DAPI (blue). **D,** Summary boxplot showing average MFI values of CD11c, CD209, CCL20, TNF, and VEGF-A expression in $CD163^+$ TAMs, and VEGF-A in tumor nests, from metastatic and nonmetastatic primary melanomas (Mann–Whitney). Scale bars, 50 μm.

unfavorable prognosis of newly diagnosed patients better than the M1/M2 polarization phenotype.

Next, we analyzed the expression of known protumoral TAM markers, such as TNF and VEGF-A (22–24), to ask whether TAMs from primary lesions display a differential cytokine profile. As with CCL20, TAMs from metastatic primary melanomas and nonmetastatic tumors expressed different amounts of both TNF and VEGF-A (Fig. 5C and D; Supplementary Fig. S4A). TNF induced CCL20 secretion by macrophages (M2 > M1) suggesting an activating mechanism, but VEGF-A did not (Supplementary Fig. S4B). In addition to TAM, VEGF-A was also detected at tumor nests in certain samples, but not restricted to metastatic primary melanomas (Fig. 5D; Supplementary Fig. S4C). TAM expression of CCL20 correlated with TAM expression of TNF and VEGF-A in patient samples ($R = 0.749$ and $R = 0.612$, respectively; Supplementary Fig. S4D). Thus, the activated state of macrophages infiltrating primary melanomas may serve as a biomarker to identify patients with high risk of metastatic spreading and poor prognosis for survival.

Discussion

CCR6/CCL20 interactions promote tumor progression in several human cancers (9, 12). Here, we showed functional CCR6 expression by human melanoma cells, whereas CCL20 induced proliferation/migration *in vitro* and enhanced tumor growth/metastasis *in vivo*. We demonstrated an association in cutaneous melanoma patients between high stromal-CCL20 expression in the primary tumor and the subsequent development of metastasis during follow-up and poorer survival. Stromal-$CCL20^{hi}$ cells were identified as TAMs, which coexpressed TNF and VEGF-A in a signature of prometastatic TAM activation.

Tumors are complex tissues that contain both malignant and nonmalignant cells, which interact to influence the progression of the tumor (25). Analysis of the molecular phenotype of each cell type requires enough tissue to separate cells, but the small specimens available from primary skin melanomas preclude such analysis. Multicolor confocal microscopy coupled to single-cell quantitative image analysis provides tools to assess protein expression by specific cell types. We quantified CCR6/CCL20 expression in primary melanomas, showing that CCR6 was expressed in tumor areas and CCL20 in stromal areas, particularly by TAMs. We also purified specific cell types to confirm that CCR6 was expressed by fresh malignant cells, whereas CCL20 was secreted by TAMs. Our results blocking human CCR6 with mAb with or without addition of exogenous CCL20 proved paracrine instead of autocrine signaling in melanoma. We conclude that there is a synergistic interaction between malignant cells and other cells in the stroma through a CCR6/CCL20 paracrine signaling loop. By contrast, in most epithelial cancers such as colorectal, lung, and pancreatic adenocarcinomas or nasopharyngeal tumors, both CCR6 and CCL20 are expressed by malignant epithelial cells, suggesting autocrine selfstimulation (11, 26–28). Paracrine signaling between stromal-CCL20 and tumoral-CCR6 may be a characteristic of certain cancers, including melanoma.

Stromal-CCL20 expression was variable across patient samples. Long-term follow-up data showed a positive association between high stromal-CCL20 levels and incidence of subsequent metastatic spreading or melanoma-specific death. In our exploratory cohort of primary melanoma patients, stromal-CCL20 expression was associated with disease-free survival by multivariate analysis, together with gender and Breslow thickness, indicating that stromal-CCL20 expression could identify patients with higher risk of metastasis. Data from independent patient series will be necessary to confirm that stromal-CCL20 is a prognostic factor for cutaneous melanoma patients. By contrast, no association was found in our study between tumoral-CCR6 expression and metastatic progression.

CCR6 was originally discovered in dendritic cells (29). A proposed immunotherapy strategy is to attract DCs to cancer sites by engineering tumors to produce CCL20 or by intratumoral injection of CCL20 (30, 31). However, CCR6 expression by malignant cells in a wide variety of tumors, including our work in melanoma, cautions against choosing this chemokine for intratumoral immunotherapy. CCL20 is the main chemokine expressed by inflamed skin (32) and mucosal epithelium (33), and it may orchestrate a protumoral inflammatory microenvironment that could promote rather than hinder tumor progression. Indeed, CCL20 from different sources recruits Treg, Th17, and Th22 cells that maintain the sort of chronic inflammation and immunosuppression that could indirectly promote cancer progression (34–36).

Studies in cancer mouse models have revealed that TAMs are heterogeneous cells that adopt a spectrum of phenotypes between the extremes M1 and M2 polarization states, including mixed M1/M2 phenotypes (37, 38). Studies identifying distinct macrophage subpopulations in human tissues are scarce (39). In one study, CD11c expression was used to identify two subpopulations of human $CD14^+$ macrophages isolated from decidua: a $CD11c^{hi}$ subset with an inflammatory phenotype, and a $CD11c^{lo}$ subset related with tissue remodeling and developmental functions (18). We used multiparametric single-cell microscopy to discriminate $CD11c^+$ DC from macrophages that coexpress other markers as CD14, CD115, and CD163. This strategy identified a minor $CCL20^{hi}$ DC2 ($CD1c^+$) subset as well as two subsets, $CD11c^{hi}$ and $CD11c^{lo}$, of macrophages in human primary melanoma tissues. We performed a correlation analysis of the coexpression of several M1/M2 markers at the single-cell level in melanoma tissues. CD11c/CD209 showed the best negative correlation, indicating that these two markers were expressed by distinct TAM subsets. Thus, we quantified M1-like, M2-like, and mixed M1/M2 subsets in human primary melanoma. We found no statistically significant differences between nonmetastatic or metastatic patients, although larger patient series may be necessary to unravel this issue. Our results reveal that $CCL20^{hi}$ expression, irrespective of M1/M2 markers, is a prometastatic TAM phenotype that is associated with poor prognosis of patients with primary cutaneous melanoma. $CCL20^{hi}$ expression may be a unique characteristic of TAMs, as neither M1 nor M2 *in vitro*–differentiated human macrophages produced substantial amounts of CCL20. We found that both tumor "conditioned" M1 and M2 macrophages upregulated CCL20 expression *in vitro*, which is consistent with the detection of $CCL20^{hi}$ expression associated with both M1 and M2 TAMs *in vivo*.

We found that macrophages from metastatic skin melanomas coexpress CCL20, TNF, and VEGF-A, which together may define the inflammatory protumoral profile of TAMs. Consistent with our results, the tumor-promoting transcriptional profile of blood monocytes from renal cell carcinoma patients showed upregulation of, among other immune-related and protumoral genes, CCL20, TNF, and VEGF-A, as well as both upregulation and downregulation of M1 and M2 gene expression (40). Expression of these genes is activated by NF-κB (41–43), which is a central regulator of macrophage function in tumors and a hallmark of tumor-promoting chronic inflammation (44). Moreover, macrophage-derived TNF has been identified as a molecular inflammatory mechanism supporting survival of melanoma cells through upregulation of the melanoma survival factor MITF (22). Together, our data suggest that inflammatory macrophages expressing CCL20, TNF, and VEGF-A promote human melanoma progression, particularly in early metastasis. The protumoral signature of macrophages identified in this work may serve to identify patients with high risk of metastasis from primary melanoma and may guide early treatments.

Disclosure of Potential Conflicts of Interest

I. Márquez-Rodas is a consultant/advisory board member for Amgen, Bioncotech, Bristol-Myers Squibb, Merck Sharp & Dohme, Novartis, Pierre Fabre, and Roche. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: R. Samaniego, A. Gutiérrez-González, P. Sánchez-Mateos

Development of methodology: R. Samaniego, A. Gutiérrez-González, A. Gutiérrez-Seijo, S. Sánchez-Gregorio, J. García-Giménez, E. Mercader, M. Relloso

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E. Mercader, I. Márquez-Rodas, J.A. Avilés, P. Sánchez-Mateos

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Samaniego, A. Gutiérrez-González, A. Gutiérrez-Seijo, I. Márquez-Rodas, M. Relloso, P. Sánchez-Mateos

Writing, review, and/or revision of the manuscript: R. Samaniego, E. Mercader, I. Márquez-Rodas, P. Sánchez-Mateos

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Samaniego, A. Gutiérrez-Seijo, P. Sánchez-Mateos

Study supervision: R. Samaniego, P. Sánchez-Mateos

Acknowledgments

This work was partially supported by the Ministry of Economy and Competitiveness ISCIII-FIS grants PI13/01454 (P. Sánchez-Mateos), PI17/01324 (P. Sánchez-Mateos and R. Samaniego), and PI16/00050 (M. Relloso), cofinanced by ERDF/FEDER Funds from the European Commission, "A way of making Europe." A. Gutiérrez-Seijo is financed by the Comunidad de Madrid YEI-program. The authors would like to thank Julia Villarejo and Raul Campos-Fernandez for expert technical assistance, Jose María Bellon for help with statistical analyses, and Amaya Puig-Kröger, Francisco Sanchez-Madrid, and David Sancho for providing reagents/antibodies.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 17, 2017; revised October 3, 2017; accepted January 9, 2018; published OnlineFirst January 23, 2018.

References

1. Chow MT, Luster AD. Chemokines in cancer. Cancer Immunol Res 2014;2:1125–31.
2. Richmond A, Yang J, Su Y. The good and the bad of chemokines/chemokine receptors in melanoma. Pigment Cell Melanoma Res 2009;22:175–86.
3. Bar-Eli M. Role of interleukin-8 in tumor growth and metastasis of human melanoma. Pathobiology 1999;67:12–8.
4. Bartolomé RA, Gálvez BG, Longo N, Baleux F, Van Muijen GN, Sánchez-Mateos P, et al. Stromal cell-derived factor-1alpha promotes melanoma cell invasion across basement membranes involving stimulation of

membrane-type 1 matrix metalloproteinase and Rho GTPase activities. Cancer Res 2004;64:2534–43.
5. Robledo MM, Bartolome RA, Longo N, Rodríguez-Frade JM, Mellado M, Longo I, et al. Expression of functional chemokine receptors CXCR3 and CXCR4 on human melanoma cells. J Biol Chem 2001;276:45098–105.
6. Longo-Imedio MI, Longo N, Treviño I, Lázaro P, Sánchez-Mateos P. Clinical significance of CXCR3 and CXCR4 expression in primary melanoma. Int J Cancer 2005;117:861–5.
7. Amersi FF, Terando AM, Goto Y, Scolyer RA, Thompson JF, Tran AN, et al. Activation of CCR9/CCL25 in cutaneous melanoma mediates preferential metastasis to the small intestine. Clin Cancer Res 2008;14:638–45.
8. Ben-Baruch A. Organ selectivity in metastasis: regulation by chemokines and their receptors. Clin Exp Metastasis 2008;25:345–56.
9. Frick VO, Rubie C, Keilholz U, Ghadjar P. Chemokine/chemokine receptor pair CCL20/CCR6 in human colorectal malignancy: an overview. World J Gastroenterol 2016;22:833–41.
10. Ghadjar P, Rubie C, Aebersold DM, Keilholz U. The chemokine CCL20 and its receptor CCR6 in human malignancy with focus on colorectal cancer. Int J Cancer 2009;125:741–5.
11. Kleeff J, Kusama T, Rossi DL, Ishiwata T, Maruyama H, Friess H, et al. Detection and localization of Mip-3alpha/LARC/Exodus, a macrophage proinflammatory chemokine, and its CCR6 receptor in human pancreatic cancer. Int J Cancer 1999;81:650–7.
12. Li Y, Huang J, Tie H, Huang H, Li H, Ren G. Prognostic significance of CCL20 and its receptor CCR6 in cancers: a meta-analysis. Int J Clin Exp Med 2016;9:1908–1919.
13. Samaniego R, Estecha A, Relloso M, Longo N, Escat JL, Longo-Imedio I, et al. Mesenchymal contribution to recruitment, infiltration, and positioning of leukocytes in human melanoma tissues. J Invest Dermatol 2013;133:2255–64.
14. Samaniego R, Palacios BS, Domiguez-Soto Á, Vidal C, Salas A, Matsuyama T, et al. Macrophage uptake and accumulation of folates are polarization-dependent in vitro and in vivo and are regulated by activin A. J Leukoc Biol 2014;95:797–808.
15. Estecha A, Sánchez-Martín L, Puig-Kröger A, Bartolomé RA, Teixidó J, Samaniego R, et al. Moesin orchestrates cortical polarity of melanoma tumour cells to initiate 3D invasion. J Cell Sci 2009;122:3492–501.
16. Barros MH, Hauck F, Dreyer JH, Kempkes B, Niedobitek G. Macrophage polarisation: an immunohistochemical approach for identifying M1 and M2 macrophages. PLoS One 2013;8:e80908.
17. Gonzalez-Dominguez E, Samaniego R, Flores-Sevilla JL, Campos-Campos SF, Gómez-Campos G, Salas A, et al. CD163L1 and CLEC5A discriminate subsets of human resident and inflammatory macrophages in vivo. J Leukoc Biol 2015;98:453–66.
18. Houser BL, Tilburgs T, Hill J, Nicotra ML, Strominger JL. Two unique human decidual macrophage populations. J Immunol 2011;186:2633–42.
19. Lumeng CN, Bodzin JL, Saltiel AR. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. J Clin Invest 2007;117:175–84.
20. Dominguez-Soto A, Sierra-Filardi E, Puig-Kröger A, Pérez-Maceda B, Gómez-Aguado F, Corcuera MT, et al. Dendritic cell-specific ICAM-3-grabbing nonintegrin expression on M2-polarized and tumor-associated macrophages is macrophage-CSF dependent and enhanced by tumor-derived IL-6 and IL-10. J Immunol 2011;186:2192–200.
21. Sica A, Mantovani A, Macrophage plasticity and polarization: in vivo veritas. J Clin Invest 2012;122:787–95.
22. Smith MP, Sanchez-Laorden B, O'Brien K, Brunton H, Ferguson J, Young H, et al. The immune microenvironment confers resistance to MAPK pathway inhibitors through macrophage-derived TNFalpha. Cancer Discov 2014;4:1214–29.
23. Kratochvill F, Neale G, Haverkamp JM, Van de Velde LA, Smith AM, Kawauchi D, et al. TNF counterbalances the emergence of M2 tumor macrophages. Cell Rep 2015;12:1902–14.
24. Laoui D, Van Overmeire E, Di Conza G, Aldeni C, Keirsse J, Morias Y, et al. Tumor hypoxia does not drive differentiation of tumor-associated macrophages but rather fine-tunes the M2-like macrophage population. Cancer Res 2014;74:24–30.
25. Egeblad M, Nakasone ES, Werb Z. Tumors as organs: complex tissues that interface with the entire organism. Dev Cell 2010;18:884–901.
26. Brand S, Olszak T, Beigel F, Diebold J, Otte JM, Eichhorst ST, et al. Cell differentiation dependent expressed CCR6 mediates ERK-1/2, SAPK/JNK, and Akt signaling resulting in proliferation and migration of colorectal cancer cells. J Cell Biochem 2006;97:709–23.
27. Nandi B, Pai C, Huang Q, Prabhala RH, Munshi NC, Gold JS. CCR6, the sole receptor for the chemokine CCL20, promotes spontaneous intestinal tumorigenesis. PLoS One 2014;9:e97566.
28. Kirshberg S, Izhar U, Amir G, Demma J, Vernea F, Beider K, Shlomai Z, et al. Involvement of CCR6/CCL20/IL-17 axis in NSCLC disease progression. PLoS One 2011;6:e24856.
29. Power CA, Church DJ, Meyer A, Alouani S, Proudfoot AE, Clark-Lewis I, et al. Cloning and characterization of a specific receptor for the novel CC chemokine MIP-3alpha from lung dendritic cells. J Exp Med 1997;186:825–35.
30. Furumoto K, Soares L, Engleman EG, Merad M. Induction of potent antitumor immunity by in situ targeting of intratumoral DCs. J Clin Invest 2004;113:774–83.
31. Bonnotte B, Crittenden M, Larmonier N, Gough M, Vile RG. MIP-3alpha transfection into a rodent tumor cell line increases intratumoral dendritic cell infiltration but enhances (facilitates) tumor growth and decreases immunogenicity. J Immunol 2004;173:4929–35.
32. Homey B, Dieu-Nosjean MC, Wiesenborn A, Massacrier C, Pin JJ, Oldham E, et al. Up-regulation of macrophage inflammatory protein-3 alpha/CCL20 and CC chemokine receptor 6 in psoriasis. J Immunol 2000;164:6621–32.
33. Tanaka Y, Imai T, Baba M, Ishikawa I, Uehira M, Nomiyama H, et al. Selective expression of liver and activation-regulated chemokine (LARC) in intestinal epithelium in mice and humans. Eur J Immunol 1999;29:633–42.
34. Walch-Ruckheim B, Mavrova R, Henning M, Vicinus B, Kim YJ, Bohle RM, et al. Stromal fibroblasts induce CCL20 through IL6/C/EBPbeta to support the recruitment of Th17 cells during cervical cancer progression. Cancer Res 2015;75:5248–59.
35. Chen KJ, Lin SZ, Zhou L, Xie HY, Zhou WH, Taki-Eldin A, et al. Selective recruitment of regulatory T cell through CCR6-CCL20 in hepatocellular carcinoma fosters tumor progression and predicts poor prognosis. PLoS One 2011;6:e24671.
36. Kryczek I, Lin Y, Nagarsheth N, Peng D, Zhao L, Zhao E, et al. IL-22(+)CD4(+) T cells promote colorectal cancer stemness via STAT3 transcription factor activation and induction of the methyltransferase DOT1L. Immunity 2014;40:772–84.
37. Van Overmeire E, Laoui D, Keirsse J, Van Ginderachter JA, Sarukhan A. Mechanisms driving macrophage diversity and specialization in distinct tumor microenvironments and parallelisms with other tissues. Front Immunol 2014;5:127.
38. Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. Cell 2010;141:39–51.
39. Gordon S, Pluddemann A, Martinez Estrada F. Macrophage heterogeneity in tissues: phenotypic diversity and functions. Immunol Rev 2014;262:36–55.
40. Chittezhath M, Dhillon MK, Lim JY, Laoui D, Shalova IN, Teo YL, et al. Molecular profiling reveals a tumor-promoting phenotype of monocytes and macrophages in human cancer progression. Immunity 2014;41:815–29.
41. Baldwin AS Jr. The NF-kappa B and I kappa B proteins: new discoveries and insights. Annu Rev Immunol 1996;14:649–83.
42. Harant H, Eldershaw SA, Lindley IJ. Human macrophage inflammatory protein-3alpha/CCL20/LARC/Exodus/SCYA20 is transcriptionally upregulated by tumor necrosis factor-alpha via a non-standard NF-kappaB site. FEBS Lett 2001;509:439–45.
43. Kiriakidis S, Andreakos E, Monaco C, Foxwell B, Feldmann M, Paleolog E. VEGF expression in human macrophages is NF-kappaB-dependent: studies using adenoviruses expressing the endogenous NF-kappaB inhibitor IkappaBalpha and a kinase-defective form of the IkappaB kinase 2. J Cell Sci 2003;116:665–74.
44. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. Cell 2010;140:883–99.